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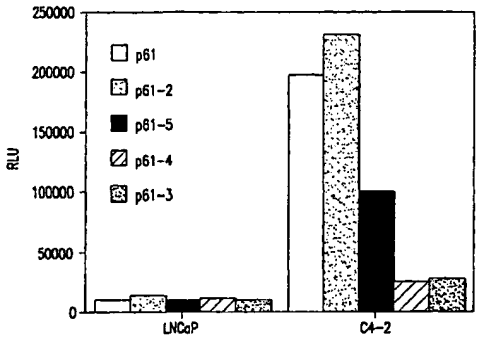

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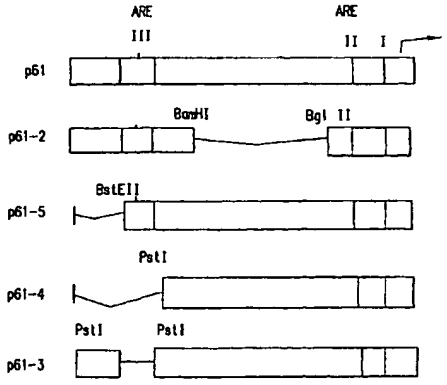

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(57) Abstract: The present invention provides methods and compositions for the delivery and expression of therapeutic genes for treating prostate and non-prostate tumors in a gene therapy setting with therapeutic genes driven by a super PSA promoter. This approach enhances the capability of increasing the size of therapeutic gene inserts and maintaining specificity and efficiency of genes expression. This form of gene therapy strategy can be applied either alone or in combination with other adjuvant therapies or used in combination with various gene therapy strategies to achieve the maximum effect in cancer treatment, and in normal and benign tissues to enhance therapeutic gains.

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GENE EXPRESSION DIRECTED BY A SUPER-PSA PROMOTER

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit under 35 U.S.C. section 119(e) of co-pending U.S. provisional application 60/162,223, filed October 29, 1999, the entire text of which is herein incorporated by reference without disclaimer.

BACKGROUND

Prostate Specific antigen (PSA) is a popular tumor marker for prostate cancer. It is synthesized and secreted mostly by the luminal epithelial cells of the human prostate (Cleutijens KBJM, Korput HAGM, et al. *Mol Endocrin* 11 (9): 1256-65). It has been reported that the serum level of PSA is well proportional to tumor size and related to the clinical stages of the disease (Gleave ME. Hsieh JT, et al. *J. Urol.* 147:1151-59). During hormonal therapy, most patients show an initial often dramatic decrease in PSA levels; however as the disease progress to hormone refractory stage, some of the patients experience a rebound of the PSA level (Montgomery BT, Young CY, et al. (1992) *The prostate* 21:63-7). Once initial hormonal therapy has failed, median survival is only 6 months (Montgomery BT, Young CY, et al. (1992) *The prostate* 21:63-7). Currently, it is poorly understood how prostate cancer progresses from an androgen dependent (AD) to an androgen independent (AI) stage. The rebound of PSA in hormone refractory prostate cancer cells and its tissue-specific expression pattern makes the PSA promoter a choice candidate for delivering therapeutic gene to prostate cancer cells.

The regulation of PSA expression in hormone-dependent and -independent prostate cancer cells is an interesting focal point. The aim of the study is to understand how hormone-dependent and independent prostate cancer cells upregulate PSA in the presence and absence of androgen stimulation respectively. The in-vitro PSA expression system used in this study consists of two cell lines: LNCaP & C4-2 (Thalmaun GN, Anizinis PE, et al. (1994) *Cancer Res.*54:2577-81); (Gleave ME. Hsieh JT, et al. (1992) *J. Urol.* 147:1151-59). Both cell lines are prostate carcinoma cells that expressed endogenous PSA and androgen receptor (AR). LNCaP represents the early stage of prostate cancer as it is hormone dependent and non-metastatic, while C4-2 represents the advanced stage of prostate cancer as it is hormone independent and highly metastatic (Thalmaun GN, Anizinis PE, et al. (1994) *Cancer Res.*54:2577-81); (Montgomery BT, Young CY, et al. (1992) *The*

prostate 21:63-7). Unlike LNCAP, C4-2 is capable of secreting a large amount of PSA in the absence of androgen (Hsieh JT, Wu HC, et al. (1993) *Cancer Res* 52:2852-57). One important aspect of this study is to define the regulation of PSA expression in hormone refractory C4-2 cells. The PSA promoter was inserted upstream to a luciferase reporter gene and transiently transfected into LNCaP and C4-2 cells. The promoter of PSA is about 6 Kb long; it contains multiple regulatory elements such as androgen-responsive element (ARE) and prostate-specific enhancer (PSE), and it is tightly regulated by androgen (Schur ER. Henderson GA, et al. *JBC* 271(12):7043-51 (1996); Rieginan PHJ, Vlietstra RJ, et al. (1991) *Mol Endocrin* 5(12):1921- 30; Cleutijens KBJM, Korput HAGM, et al. *Mol Endocrin* (1997) 11:148-161). Promoter analysis studies were done to identify the elements within the promoter that is critical for the expression of PSA in the absence of androgen. Two distinct elements were found to contribute greatly to the PSA promoter activity in hormone refractory C4-2 cells. When these two elements were put together, it surpasses the native PSA promoter activity by 2-4 fold, yet still retains the tissue specificity of the native PSA promoter. The ultimate aim of the study is to identify the transcription factors that interact with these two regulatory elements.

SUMMARY OF THE INVENTION

Previously, it has been shown that the 632 bp proximal PSA promoter could not direct prostate specificity in transgenic mice, only the full length PSA promoter exhibits tissue specificity in vivo (Schur ER. Henderson GA, et al. (1996) *JBC* 271(12):7043-51). Full length PSA promoter has also been used for the delivery and expression of therapeutic genes in prostate cancer cells and tumor xenografts. Obviously, certain specific elements in the PSA promoter control the prostate specificity of gene expression. By manipulating the native PSA promoter, Henderson's group have generated a smaller version of PSA promoter PSE for gene therapy purpose (Yeh S, Chang C. (1996) *PNAS* 93:5517-21). PSE contains the proximal 200 bp PSA promoter and the 440bp ARE enhancer core (AREc) element that was identified by Trapman (Stamey TA, Yang N, et al. (1989) *J Urol.* 141:1088-90). However, PSE is not prostate tissue specific (unpublished data) and its activity is somewhat lower than the native PSA promoter.

A super-PSA promoter was created which has 2-4 fold higher basal activity than the native PSA promoter. Moreover, the super-PSA promoter is also highly inducible by androgen in a prostate tissue-specific manner. This promoter is generated by juxtaposing the ARE enhance core with a newly discovered 120bp element pTATA. The basal activity

of the super PSA promoter is especially impressive in prostate cells that express AR (such as LNCaP, C4-2 & NbE). In cells with neither AR nor PSA expression (such as PC-3), super PSA promoter activity is still 2-4 fold higher than the native PSA promoter. When comparing the super PSA promoter activity between AD LNCaP and Al C4-2, C4-2 clearly could activate the super PSA. promoter to a much higher level than LNCaP in the absence of androgen stimulation. Thus, super PSA promoter is a strong promoter to drive gene expression in hormone refractory tumor cells. The high basal activity of the super PSA promoter together with its great inducibility by androgen make super PSA promoter a highly efficient promoter to drive genes expression both in the absence and presence of androgen in cells.

Additionally, the size of the super-PSA promoter (560bp) is significantly smaller than the native PSA promoter (6Kb),which provides advantages for transgenic studies, gene therapy applications and identification of cis-acting and tissue-specific transcription factors for conferring AR-mediated action.

Within the pTATA element, a P2 region was discovered that is essential for the pTATA activity. DNA footprinting was done with the 120bp pTATA, 2 distinct sites were protected by protein factor(s) from DnaseI digestion. P2 was then identified as one of the sites. It has been suggested that a transcription factor or factors specific for a PSA producing cell interacts with P2 site and activates the promoter activity. This P2 region is of high potential, for it could be used either alone or in conjunction with other promoter elements to generate chimeric promoter constructs for the delivery and expression of genes in normal and tumor cells with high degree of specificity and efficiency. Moreover, by identifying a specific transcription factor that interacts with the P2 site, it could allow the expression of genes in cells without AR nor expressing PSA (such as PC-3). Data is provided to show that when 3 copies of P2 inserted upstream of an artificial TATA box, it could drive high expression of the luciferase reporter gene. The activity of this 3(P2) construct actually surpass the wild type PSA promoter activity in LNCaP cells. Thus, multiple copies of P2 works well in prostate cells with endogenous AR and PSA. After identifying the cis-acting factor(s) of P2, then a P2-containing promoter together with the cis-acting factor could then be delivered into any cells (including cells that do not produce PSA) to trigger positive regulatory responses.

This concept of specific interaction between a transcription factor and a P2 site or P2-like sequences could exist in another region around the androgen response element III (AREIII) within AREc. It is believed that prostate specific factor(s) interacts with AR binds to specific DNA sequences (e.g. P2 or P2-like consensus sequences), and transactivates the gene expression in PSA producing cells. Therefore, the regions around

AREIII could also be used with other promoter elements to generate chimeric constructs that could be used to direct gene expression in a ligand-dependant or -independent and tissue-specific manner.

BRIEF DESCRIPTION OF THE DRAWINGS:

FIG. 1 Summary of Promoter analysis studies.

FIG. 2 A comparison of the super PSA promoter (AREc/pTATA) activity with the native PSA promoter (p61) and the PSE promoter (AREc/(PA8).

FIG. 3 The complete sequence of the super-PS A promoter.

FIG. 4 The binding of the potential protein factors to the radiolabeled P2 element in an Electrophoretic~Mobility-Shift-Assay (EMSA).

FIG. 5 Determination of the basal activity of the Super-PSA promoter and the native PSA promoter in other prostate cell lines.

FIG. 6 Androgen inducibility of the native and super PSA promoter in NbE & PC-3 cells.

DETAILED DESCRIPTION OF THE INVENTION

PSA is a single chain glycoprotein with a molecular weight of approximately 33 kDa. It is a member of the family of human kallikrein-like serine proteases (Landwall A. (1989). *Biochem. Biophys. Res. Commun.* 161:1151-59); (Lilja H. (1985). *J. Clin. Invest.* 76:1899-1903). PSA is synthesized in and secreted by the luminal epithelial cells of the human prostate. In vivo, PSA may function to liquify the seminal coagulum by proteolysis of seminogelin and fibronectin (Cleutijens KBJM, Korput HAGM, et al. *Mol Endocrin* 11 (Stamey TA, Yang N, et al. *J Urol.* 141:1088-90): 1256-65). The PSA gene consists of five exons, and spans a region of approximately 5Kb (Nazarth LV, Wigel NL. (1996). *JBC* 271:19900-19907). The gene is clustered with the glandular kallikrein (hGK-1) and renal kallikrein (KLK-l) genes, in an area of 60 Kb on human chromosome 19q13.2-l3.4 (Nazarth

LV, Wigel NL. (1996). *JBC* 271:19900-19907). PSA is almost exclusively expressed in the human prostate and prostate derived tumors and tumor cell lines. Gleave ME. Hsieh JT, et al. (1992) *J. Urol.* 147:1151-59. Therefore, PSA is the mostly widely used serum marker for the diagnosis and management of prostate cancer. Among the gene products specifically expressed by the human prostate, so far only the transcription regulation of the PSA gene has been studied (Schur ER. Henderson GA, et al. *JBC* 271(12):7043-51. 1996); (Rieginan PHJ, Vlietstra RJ, et al. (1991) *Mol Endocrin* 5(12): 1921- 30); (Cleutijens KBJM, Korput HAGM, et al. *Mol Endocrin* (1997) 11:148-161). PSA promoter is about 6 Kb long, it is tightly regulated by androgen through the three androgen responsive elements (AREs) in the promoter (Rieginan PHJ, Vlietstra RJ, et al. (1991) *Mol Endocrin* 5(12): 1921- 30); (Cleutijens KBJM, Korput HAGM, et al. *Mol Endocrin* (1997) 11:148-161). Upon binding to androgen, ARs translocate into the nucleus and bind to the AREs which will then activate the PSA gene expression. Two of AREs are located in the proximal PSA promoter at positions -170 (ARE I:AGAACAgcaAGTGCT) and -394 (ARE II: GGATCAgggAGTCTC). The third ARE-ARE III is located at ~4200 bp upstream of the transcription start site. All three ARES contribute to the maximal androgen inducibility of the PSA promoter. ARE III was shown to be part of a very strong androgen regulated enhancer region (AREc). In Western countries, adenocarcinoma of the prostate is the most frequently diagnosed tumor in men, and one of the leading causes of male cancer death (Culig Z, Hobisch A, et al. (1994). Cancer Res. 54:5474-781). Therapeutic approaches for prostate cancer depend on the stage of the malignancy. Initially, the growth of the majority of prostate tumors depends, like normal prostate development, on androgens (Ruiter de PE, Twuwen R. et al. (1995) *Mol Cell endocrinol* 110:R1-6). Therefore therapy of metastasized tumors is generally based upon androgen ablation or blockade of AR function. After onset of endocrine therapy, most prostate tumors show regression (Stamey TA, Yang N, et al. (1989) *J Urol.* 141:1088-90); (Lyss AP. (1987) "Systemic treatment for prostate cancer." American J Med 83:1120-27). However, essentially all originally hormone responsive tumors become apparently hormone-independent during time (Thalmaun GN, Anizinis PE, et al. (1994) *Cancer Res.* 54:2577-81). PSA is expressed in the vast majority of prostate cancers, increase of serum PSA during endocrine therapy is generally considered as evidence for recurrence or progression of prostate tumor (Thalmaun GN, Anizinis PE, et al. (1994) *Cancer Res.* 54:2577-81). The upregulation of PSA in hormone-independent tumor cells may be due to ligand-independent activation of the AR. In prostate cancer cells, Culig et al (Kleinerman DI Troncoso P, et al. (1996). *AUA ninety-first annual meeting, Orlando, J. Urol*) has shown growth factors like IGF-1 and KGF could induce AR mediated, promoter specific transcriptional activation. Furthermore, cross-talk between AR

and both the PKA or PKC-signalling pathways has been reported (Nazarth LV, Wigel NL. (1996). *JBC* 271:19900-19907); (Ruiter de PE, Twuwen R. et al. (1995) *Mol Cell endocrinol* 110:R1-6). The role of AR in prostate cancer is still controversial. The majority of locally progressive, hormone refractory tumors show high AR expression, although more heterogeneous than in the normal prostate (Landwall A. (1989). *Biochem. Biophys. Res. Commun.* 161:1151-59). Interestingly, distant prostatic carcinoma metastases in bone express AR and the expression level seems even higher and more homogeneous than in locally recurrent tumor (Landwall A. (1989). *Biochem. Biophys. Res. Commun.* 161:1151-59). An increased level of AR expression could allow cancer cells to compensate for their growth and survival under androgen-deprived conditions and to continue their proliferation in the presence of a low level of serum testosterone.

The concept of delivery and expression of therapeutic genes to tumor cells through the use of tissue-specific promoters has been well recognized. This approach decreases the adverse effects of the therapeutic genes on normal cells and increases the specificity and efficiency of gene transfer to normal and tumor cells. Due to its highly tissue-specific expression pattern of PSA in normal, hyperpfastic and malignant prostate epithelia researchers have been trying to use the PSA promoter to direct tissue-specific therapeutic gene expression in prostate cancer cells. The obstacles facing the use of native PSA promoter to direct the specific and efficient gene expression in prostate cells are: 1) Full-length PSA promoter alone without the administration of androgen may not be strong enough to drive the expression of down-stream therapeutic genes in prostate cancer cells. 2) PSA promoter is relatively large that could decrease the capability of genes inserted for therapeutic purposes. These obstacles can be overcome by the following strategies which are described in this invention. First, a super PSA promoter has been generated by juxtaposing AREc enhancer element and pTATA element. This super PSA promoter has 2-4 fold higher activity than the native PSA promoter, and it is highly inducible by androgen in a tissue-specific manner. Moreover, the activity of the super PSA promoter is especially strong in hormone-independent prostate cancer cells which makes it a superior promoter in delivering genes into hormone-independent prostate cancer cells. Additionally, through DNA footprinting experiment, a P2 element has been identified that is essential for the PSA promoter activity. The factor(s) that binds to P2 element may be specific to cells which are capable of expressing endogenous PSA. By manipulating the P2 element, either using multiple copies of it or altering the sequence in the site, the PSA promoter or other tissue-specific promoter activity can be enhanced in normal, benign and malignant prostate cells. The assumption is based upon the observation that when 3 copies of P2 element were used to drive the expression of a reporter gene, tremendous activity is observed in the absence of

androgen. Therefore, tandem copies of P2 are able to enhance the activity of a promoter in the PSA-positive cells (e.g. LNCaP and C4-2). Since the interaction of P2 element with its cis-acting factor(s) do not require neither androgen nor AR, it is conceivable that a P2 element containing promoter could deliver therapeutic genes effectively even in PSA- and AR- negative prostate cells (e.g. PC-3), with provision that the P2 cis-acting factor is also introduced into the target cells. Even though the cis-acting factor may be specific for PSA producing cells, the use of P2 element in gene therapy is not limited to prostate cells. It is anticipated that by introducing the cis-acting factor together with the P2 site containing construct, it would be possible to deliver therapeutic genes in any kind of cells. Therefore, by identifying the P2 cis-acting factor(s), P2 element would become a very flexible and strong promoter to deliver therapeutic genes in virtually any given cell.

In summary, the present invention provides methods and compositions for the delivery and expression of therapeutic genes for treating prostate and non-prostate tumors in a gene therapy setting with therapeutic genes driven by a super PSA promoter. It is anticipated that the pTATA and P2 box alone also direct therapeutic gene expression in prostate and non-prostate cells for therapeutic gains. These approaches enhance the capability of increasing the sizes of therapeutic gene inserts and maintaining specificity and efficiency of genes expression. This form of gene therapy strategy can be applied either alone or in combination with other adjuvant therapies or used in combination with various gene therapy strategies to achieve the maximum effect in cancer treatment, and in normal and benign tissues to enhance therapeutic gains.

1 ADDITIONAL EMBODIMENTS OF THE INVENTION

In additional embodiments, the invention disclosed herein provides a model for prostate-specific gene transcription. The invention is based in part on the functional characterization described herein of a super-PSA regulatory region, which is highly inducible by androgen and is expressed in a prostate-specific manner.

The present invention provides compositions and methods for screening compounds that modulate expression within prostate cells. In particular, it provides compositions comprising nucleotides from a PSA promoter, and transcriptionally active fragments thereof, as well as nucleic acids that hybridize under highly stringent and moderately stringent conditions to such nucleotides, that control the expression of nucleic acid coding sequences in a prostate specific manner. Specifically provided are expression vectors comprising the super-PSA regulatory region, and transcriptionally active fragments thereof, operably associated to a heterologous reporter gene, *e.g.*, LacZ, and host cells and

transgenic animals containing such vectors. The invention also provides methods for using such vectors, cells and animals for screening candidate molecules for agonists and antagonists of prostate-related disorders. Methods for using molecules and compounds identified by the screening assays for therapeutic treatments also are provided.

For example, and not by way of limitation, a composition comprising a reporter gene is operatively linked to a prostate specific regulatory sequence, herein called the super-PSA regulatory region. The super-PSA driven reporter gene is expressed as a transgene in animals. The transgenic animal, and cells derived from the prostate of such transgenic animal, can be used to screen compounds for candidates useful for modulating prostate-related disorders. Without being bound by any particular theory, such compounds are likely to interfere with the function of trans-acting factors, such as transcription factors, cis-acting elements, such as promoters and enhancers, as well as any class of post-transcriptional, translational or post-translational compounds involved in prostate-related disorders. As such, they are powerful candidates for treatment of such disorders.

In one embodiment, the invention provides methods for high throughput screening of compounds that modulate specific expression of genes within the prostate. In this aspect of the invention, cells from the prostate are removed from the transgenic animal and cultured *in vitro*. The expression of the reporter gene is used to monitor prostate-specific gene activity. In a specific embodiment, LacZ is the reporter gene. Compounds identified by this method can be tested further for their effect on prostate-related disorders in normal animals.

In another embodiment, the transgenic animal models of the invention can be used for *in vivo* screening to test the mechanism of action of candidate drugs for their effect on prostate-related disorders. Specifically, the effects of the drugs on prostate-related disorders can be assayed.

In another embodiment, a gene therapy method for treating and/or preventing prostate-related disorders is provided. Super-PSA regulatory sequences are used to drive prostate-specific expression of therapeutic molecules and introduced in the cells of the prostate. The method comprises introducing a super-PSA regulatory sequence operatively associated with a nucleic acid encoding a therapeutic molecule into cells of the prostate. In one embodiment, the invention provides a preventative gene therapy method comprising introducing a super-PSA regulatory sequence operatively associated with a nucleic acid encoding a therapeutic molecule into cells of the prostate to delay and/or prevent a prostate-related disorder. In a specific embodiment, the invention provides a gene therapy method for treatment of cancer or other proliferative disorder, including, but not limited to prostate cancer. The super-PSA regulatory sequence is used to direct the expression of one or more

coding sequences specifically in the tumor cells of a patient.

The invention further provides methods for screening for novel transcription factors that modulate the super-PSA regulatory sequence. Such novel transcription factors identified by this method can be used as targets for treating prostate-related disorders.

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1.1 Polynucleotides and Nucleic Acids of the Invention

The present invention encompasses polynucleotide sequences comprising 5' regulatory regions, and transcriptionally active fragments thereof, of the PSA gene. In particular, the present invention provides a polynucleotide comprising the pTATA sequence depicted in Figure 3 that is located within the PSA gene, and transcriptionally active fragments thereof. The pTATA sequence contains *cis* elements required to direct prostate-specific transcription *in vivo*. For example, a 23 bp fragment (P2 region of pTATA) of the pTATA sequence is essential for pTATA activity. The present invention further encompasses a super-PSA regulatory region which comprises the pTATA nucleotide sequence depicted in Figure 3 juxtaposed to the AREc nucleotide sequence depicted in Figure 3. This super-PSA regulatory region is sufficient to confer 2-4 fold higher basal activity than the native PSA promoter, and it is highly inducible by androgen in a prostate-specific manner.

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The invention further provides probes, primers and fragments of the super-PSA regulatory region. In one embodiment, purified nucleic acids consisting of at least 8 nucleotides (*i.e.*, a hybridizable portion) of a super-PSA regulatory sequence are provided; in other embodiments, the nucleic acids consist of at least 20 (contiguous) nucleotides, 25 nucleotides, 50 nucleotides, 100 nucleotides, 200 nucleotides or 500 nucleotides of a super-PSA sequence. Methods which are well known to those skilled in the art can be used to construct these sequences, either in isolated form or contained in expression vectors. These methods include, for example, *in vitro* recombinant DNA techniques, synthetic techniques and *in vivo* genetic recombination. See, *e.g.*, the techniques described in Sambrook *et al.*, 1989, supra, and Ausabel *et al.*, 1989, *supra*; also see the techniques described in "Oligonucleotide Synthesis", 1984, Gait M.J. ed., IRL Press, Oxford, which is incorporated herein by reference in its entirety.

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In another embodiment, the nucleic acids are smaller than 20, 25, 35, 200 or 500 nucleotides in length. Nucleic acids can be single or double stranded. The invention also encompasses nucleic acids hybridizable to or complementary to the foregoing sequences. In specific aspects, nucleic acids are provided which comprise a sequence complementary to at least 10, 20, 25, 50, 100, 200, 500 nucleotides or the entire regulatory

region of a super-PSA sequence.

The probes, primers and fragments of the super-PSA regulatory region provided by the present invention can be used by the research community for various purposes. They can be used as molecular weight markers on Southern gels; as chromosome markers or tags (when labeled) to identify chromosomes or to map related gene positions; to compare with endogenous DNA sequences in patients to identify potential genetic disorders; as probes to hybridize and thus discover novel, related DNA sequences; as a source of information to derive PCR primers for genetic fingerprinting; and as a probe to "subtract-out" known sequences in the process of discovering other novel polynucleotides. Methods for performing the uses listed above are well known to those skilled in the art. References disclosing such methods include, without limitation, "Molecular Cloning: A Laboratory Manual", 2d ed., Cold Spring Harbor Laboratory Press, Sambrook, J., E. F. Fritsch and T. Maniatis eds., 1989, and "Methods in Enzymology: Guide to Molecular Cloning Techniques", Academic Press, Berger, S. L. and A. R. Kimmel eds., 1987.

The nucleotide sequences of the invention also include nucleotide sequences that have at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98% or more nucleotide sequence identity to the nucleotide sequences depicted in Figure 3, and/or transcriptionally active fragments thereof.

To determine the percent identity of two amino acid sequences or of two nucleic acids, the sequences are aligned for optimal comparison purposes (*e.g.*, gaps can be introduced in the sequence of a first amino acid or nucleic acid sequence for optimal alignment with a second amino or nucleic acid sequence). The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (*i.e.*, % identity = # of identical overlapping positions/total # of positions x 100). In one embodiment, the two sequences are the same length.

The determination of percent identity between two sequences also can be accomplished using a mathematical algorithm. A preferred, non-limiting example of a mathematical algorithm utilized for the comparison of two sequences is the algorithm of Karlin and Altschul (1990) *Proc. Natl. Acad. Sci. USA 87*:2264-2268, modified as in Karlin and Altschul (1993) *Proc. Natl. Acad. Sci. USA 90*:5873-5877. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul, *et al.* (1990) *J. Mol. Biol. 215*:403-410. BLAST nucleotide searches can be performed with the NBLAST

program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to a nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to a protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.* (1997) *Nucleic Acids Res.25*:3389-3402. Alternatively, PSI-Blast can be used to perform an iterated search which detects distant relationships between molecules (*Id.*). When utilizing BLAST, Gapped BLAST and PSI-Blast programs, the default parameters of the respective programs (*e.g.*, XBLAST and NBLAST) can be used (see http://www.ncbi.nlm.nih.gov). Another preferred, non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller, (1988) *CABIOS 4*:11-17. Such an algorithm is incorporated into the ALIGN program (version 2.0) which is part of the GCG sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4 can be used. In an alternate embodiment, alignments can be obtained using the NA_MULTIPLE_ALIGNMENT 1.0 program, using a GapWeight of 5 and a GapLengthWeight of 1.

The percent identity between two sequences can be determined using techniques similar to those described above, with or without allowing gaps. In calculating percent identity, typically only exact matches are counted.

The invention also encompasses:

(a) DNA vectors that contain any of the foregoing super-PSA regulatory sequences and/or their complements (*i.e.*, antisense);

(b) DNA expression vectors that contain any of the foregoing super-PSA regulatory element sequences operatively associated with a heterologous gene, such as a reporter gene; and

(c) genetically engineered host cells that contain any of the foregoing super-PSA regulatory element sequences operatively associated with a heterologous gene such that the super-PSA regulatory element directs the expression of the heterologous gene in the host cell.

Also encompassed within the scope of the invention are various transcriptionally active fragments of this regulatory region. A "transcriptionally active" or "transcriptionally functional" fragment of a super-PSA sequence according to the present invention refers to a polynucleotide comprising a fragment of said polynucleotide which is functional as a regulatory region for expressing a recombinant polypeptide or a recombinant polynucleotide in a recombinant cell host. For the purpose of the invention, a nucleic acid

or polynucleotide is "transcriptionally active" as a regulatory region for expressing a recombinant polypeptide or a recombinant polynucleotide if said regulatory polynucleotide contains nucleotide sequences which contain transcriptional information, and such sequences are operably associated to nucleotide sequences which encode the desired polypeptide or the desired polynucleotide.

In particular, the transcriptionally active fragments of the super-PSA regulatory region of the present invention encompass those fragments that are of sufficient length to promote transcription of a heterologous gene, such as a reporter gene, when operatively linked to the super-PSA regulatory sequence and transfected into a prostate cell line. Typically, the regulatory region is placed immediately 5' to, and is operatively associated with the coding sequence. As used herein, the term "operatively associated" refers to the placement of the regulatory sequence immediately 5' (upstream) of the reporter gene, such that trans-acting factors required for initiation of transcription, such as transcription factors, polymerase subunits and accessory proteins, can assemble at this region to allow RNA polymerase dependent transcription initiation of the reporter gene.

In one embodiment, the polynucleotide sequence chosen may further comprise other nucleotide sequences, either from the PSA gene, or from a heterologous gene. In another embodiment, multiple copies of a promoter sequence, or a fragment thereof, may be linked to each other. For example, the promoter sequence, or a fragment thereof, may be linked to another copy of the promoter sequence, or another fragment thereof, in a head to tail, head to head, or tail to tail orientation. In another embodiment, a prostate cell-specific enhancer may be operatively linked to the super-PSA regulatory sequence, or fragment thereof, and used to enhance transcription from the construct containing the super-PSA regulatory sequence.

Also encompassed within the scope of the invention are modifications of this nucleotide sequence without substantially affecting its transcriptional activities. Such modifications include additions, deletions and substitutions. In addition, any nucleotide sequence that selectively hybridizes to the complement of the sequence depicted in Figure 3 under stringent conditions, and is capable of activating the expression of a coding sequence is encompassed by the invention. Exemplary moderately stringent hybridization conditions are as follows: prehybridization of filters containing DNA is carried out for 8 hours to overnight at 65°C in buffer composed of 6X SSC, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.02% BSA, and 500 µg/mℓ denatured salmon sperm DNA. Filters are hybridized for 48 hours at 65°C in prehybridization mixture containing 100 µg/mℓ denatured salmon sperm DNA and 5-20 X 10^6 cpm of ^{32}P-labeled probe. Washing of filters is done at 37°C for 1 hour in a solution containing 2X SSC, 0.01% PVP, 0.01%

Ficoll, and 0.01% BSA. This is followed by a wash in 0.1X SSC at 50°C for 45 min before autoradiography. Alternatively, exemplary conditions of high stringency are as follows: *e.g.*, hybridization to filter-bound DNA in 0.5 M $NaHPO_4$, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65°C, and washing in 0.1xSSC/0.1% SDS at 68°C (Ausubel F.M. et al., eds., 1989, Current Protocols in Molecular Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley & sons, Inc., New York, at p. 2.10.3). Other conditions of high stringency which may be used are well known in the art. In general, for probes between 14 and 70 nucleotides in length the melting temperature (TM) is calculated using the formula: Tm(°C)=81.5+16.6(log[monovalent cations (molar)])+0.41 (% G+C)-(500/N) where N is the length of the probe. If the hybridization is carried out in a solution containing formamide, the melting temperature is calculated using the equation Tm(°C)=81.5+16.6(log[monovalent cations (molar)])+0.41(% G+C)-(0.61% formamide)-(500/N) where N is the length of the probe. In general, hybridization is carried out at about 20-25 degrees below Tm (for DNA-DNA hybrids) or 10-15 degrees below Tm (for RNA-DNA hybrids).

The super-PSA regulatory region, or transcriptionally functional fragments thereof, is preferably derived from a mammalian organism. Screening procedures which rely on nucleic acid hybridization make it possible to isolate gene sequences from various organisms. The isolated polynucleotide sequence disclosed herein, or fragments thereof, may be labeled and used to screen a cDNA library constructed from mRNA obtained from appropriate cells or tissues (*e.g.*, prostate tissue) derived from the organism of interest. The hybridization conditions used should be of a lower stringency when the cDNA library is derived from an organism different from the type of organism from which the labeled sequence was derived. Low stringency conditions are well know to those of skill in the art, and will vary depending on the specific organisms from which the library and the labeled sequence are derived. For guidance regarding such conditions see, for example, Sambrook *et al.*, 1989, Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Press, N.Y., and Ausabel *et al.*, 1989, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y., each of which is incorporated herein by reference in its entirety. Further, mammalian super-PSA regulatory region homologues may be isolated from, for example, bovine or other non-human nucleic acid, by performing polymerase chain reaction (PCR) amplification using two primer pools designed on the basis of the nucleotide sequence of the super-PSA regulatory region disclosed herein. The template for the reaction may be cDNA obtained by reverse transcription of the mRNA prepared from, for example, bovine or other non-human cell lines, or tissue known to express the PSA gene. For guidance regarding such conditions, see, *e.g.*, Innis *et al.* (Eds.)

1995, *PCR Strategies*, Academic Press Inc., San Diego; and Erlich (ed) 1992, *PCR Technology*, Oxford University Press, New York, each of which is incorporated herein by reference in its entirety.

Promoter sequences within the 5' non-coding regions of the PSA gene may be further defined by constructing nested 5' and/or 3' deletions using conventional techniques such as exonuclease III or appropriate restriction endonuclease digestion. The resulting deletion fragments can be inserted into the promoter reporter vector to determine whether the deletion has reduced or obliterated promoter activity, such as described, for example, by Coles *et al.* (Hum. Mol. Genet., 7:791-800, 1998). In this way, the boundaries of the promoters may be defined. If desired, potential individual regulatory sites within the promoter may be identified using site directed mutagenesis or linker scanning to obliterate potential transcription factor binding sites within the promoter individually or in combination. The effects of these mutations on transcription levels may be determined by inserting the mutations into cloning sites in promoter reporter vectors. These types of assays are well known to those skilled in the art (WO 97/17359, US 5,374,544, EP 582 796, US 5,698,389, US 5,643,746, US5,502,176, and US 5,266,488).

The super-PSA regulatory regions and transcriptionally functional fragments thereof, and the fragments and probes described herein which serve to identify super-PSA regulatory regions and fragments thereof, may be produced by recombinant DNA technology using techniques well known in the art. Methods which are well known to those skilled in the art can be used to construct these sequences, either in isolated form or contained in expression vectors. These methods include, for example, *in vitro* recombinant DNA techniques, synthetic techniques and *in vivo* genetic recombination. See, *e.g.,* the techniques described in Sambrook *et al.*, 1989, supra, and Ausabel *et al.*, 1989, *supra*; also see the techniques described in "Oligonucleotide Synthesis", 1984, Gait M.J. ed., IRL Press, Oxford, which is incorporated herein by reference in its entirety.

Alterations in the regulatory sequences can be generated using a variety of chemical and enzymatic methods which are well known to those skilled in the art. For example, regions of the sequences defined by restriction sites can be deleted. Oligonucleotide-directed mutagenesis can be employed to alter the sequence in a defined way and/or to introduce restriction sites in specific regions within the sequence. Additionally, deletion mutants can be generated using DNA nucleases such as Bal31, ExoIII, or S1 nuclease. Progressively larger deletions in the regulatory sequences are generated by incubating the DNA with nucleases for increased periods of time (see, *e.g.*, Ausubel *et al.*, 1989, *supra*).

The altered sequences are evaluated for their ability to direct expression of

heterologous coding sequences in appropriate host cells. It is within the scope of the present invention that any altered regulatory sequences which retain their ability to direct expression of a coding sequence be incorporated into recombinant expression vectors for further use.

1.2 Analysis of Prostate-Specific Promoter Activity

The super-PSA regulatory region shows selective tissue and cell-type specificity; *i.e.*, it induces gene expression in a prostate-specific manner. Thus, the regulatory region, and transcriptionally active fragments thereof, of the present invention may be used to induce expression of a heterologous coding sequence in prostate cells. The present invention provides for the use of the super-PSA regulatory region to achieve tissue specific expression of a target gene. The activity and the specificity of the super-PSA regulatory region can further be assessed by monitoring the expression level of a detectable polynucleotide operably associated with the super-PSA regulatory region in different types of cells and tissues. As discussed hereinbelow, the detectable polynucleotide may be either a polynucleotide that specifically hybridizes with a predefined oligonucleotide probe, or a polynucleotide encoding a detectable protein.

1.2.1 Super-PSA Promoter Driven Reporter Constructs

The regulatory polynucleotides according to the invention may be advantageously part of a recombinant expression vector that may be used to express a coding sequence, or reporter gene, in a desired host cell or host organism. The super-PSA regulatory region of the present invention, and transcriptionally active fragments thereof, may be used to direct the expression of a heterologous coding sequence. In particular, the present invention encompasses mammalian super-PSA regulatory regions. In accordance with the present invention, transcriptionally active fragments of the super-PSA regulatory region encompass those fragments of the region which are of sufficient length to promote transcription of a reporter coding sequence to which the fragment is operatively linked.

A variety of reporter gene sequences well known to those of skill in the art can be utilized, including, but not limited to, genes encoding fluorescent proteins such as green fluorescent protein (GFP), enzymes (*e.g.* CAT, beta-galactosidase, luciferase) or antigenic markers. For convenience, enzymatic reporters and light-emitting reporters analyzed by colorometric or fluorometric assays are preferred for the screening assays of the

invention.

In one embodiment, for example, a bioluminescent, chemiluminescent or fluorescent protein can be used as a light-emitting reporter in the invention. Types of light-emitting reporters, which do not require substrates or cofactors, include, but are not limited to the wild-type green fluorescent protein (GFP) of *Victoria aequoria* (Chalfie *et al.*, 1994, Science 263:802-805), and modified GFPs (Heim *et al.*, 1995, Nature 373:663-4; PCT publication WO 96/23810). Transcription and translation of this type of reporter gene leads to the accumulation of the fluorescent protein in test cells, which can be measured by a fluorimeter, or a flow cytometer, for example, by methods that are well known in the art (see, *e.g.*, Lackowicz, 1983, Principles of Fluorescence Spectroscopy, Plenum Press, New York).

Another type of reporter gene that may be used are enzymes that require cofactor(s) to emit light, including but not limited to, Renilla luciferase. Other sources of luciferase also are well known in the art, including, but not limited to, the bacterial luciferase (*luxAB* gene product) of *Vibrio harveyi* (Karp, 1989, Biochim. Biophys. Acta 1007:84-90; Stewart *et al.* 1992, J. Gen. Microbiol, 138:1289-1300), and the luciferase from firefly, *Photinus pyralis* (De Wet *et al.* 1987, Mol. Cell. Biol. 7:725-737), which can be assayed by light production (Miyamoto *et al.*, 1987, J. Bacteriol. 169:247-253; Loessner *et al.* 1996, Environ. Microbiol. 62:1133-1140; and Schultz & Yarus, 1990, J. Bacteriol. 172:595-602).

Reporter genes that can be analyzed using colorimetric analysis include, but are not limited to, β-galactosidase (Nolan *et al.* 1988, Proc. Natl. Acad. Sci. USA 85:2603-07), β-glucuronidase (Roberts *et al.* 1989, Curr. Genet. 15:177-180), luciferase (Miyamoto *et al.*, 1987, J. Bacteriol. 169:247-253), or β-lactamase. In one embodiment, the reporter gene sequence comprises a nucleotide sequence which encodes a *LacZ* gene product, β-galactosidase. The enzyme is very stable and has a broad specificity so as to allow the use of different histochemical, chromogenic or fluorogenic substrates, such as, but not limited to, 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-gal), lactose 2,3,5-triphenyl-2H-tetrazolium (lactose-tetrazolium) and fluorescein galactopyranoside (see Nolan *et al.*, 1988, *supra*).

In another embodiment, the product of the *E. coli* β-glucuronidase gene (GUS) can be used as a reporter gene (Roberts *et al.* 1989, Curr. Genet. 15:177-180). GUS activity can be detected by various histochemical and fluorogenic substrates, such as X-glucuronide (Xgluc) and 4-methylumbelliferyl glucuronide.

In addition to reporter gene sequences such as those described above, which provide convenient colorimetric responses, other reporter gene sequences, such as, for

example, selectable reporter gene sequences, can routinely be employed. For example, the coding sequence for chloramphenicol acetyl transferase (CAT) can be utilized, leading to super-PSA regulatory region-dependent expression of chloramphenicol resistant cell growth. The use of CAT and the advantages of a selectable reporter gene are well known to those skilled in the art (Eikmanns *et al.* 1991, *Gene* 102:93-98). Other selectable reporter gene sequences also can be utilized and include, but are not limited to, gene sequences encoding polypeptides which confer zeocin (Hegedus *et al.* 1998, *Gene* 207:241-249) or kanamycin resistance (Friedrich & Soriano, 1991, *Genes. Dev.* 5:1513-1523).

Other reporter genes, such as toxic gene products, potentially toxic gene products, and antiproliferation or cytostatic gene products, also can be used. In another embodiment, the detectable reporter polynucleotide may be either a polynucleotide that specifically hybridizes with a predefined oligonucleotide probe, or a polynucleotide encoding a detectable protein, including a PSA polypeptide or a fragment or a variant thereof. This type of assay is well known to those skilled in the art (US 5,502,176 and US 5,266,488).

Super-PSA driven reporter constructs can be constructed according to standard recombinant DNA techniques (see, *e.g.*, *Methods in Enzymology*, 1987, volume 154, Academic Press; Sambrook *et al.* 1989, Molecular Cloning - A Laboratory Manual, 2nd Edition, Cold Spring Harbor Press, New York; and Ausubel *et al.* Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, New York, each of which is incorporated herein by reference in its entirety).

Methods for assaying promoter activity are well-known to those skilled in the art (see, *e.g.*, Sambrook *et al.*, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989). An example of a typical method that can be used involves a recombinant vector carrying a reporter gene and sequences from a PSA gene. Briefly, the expression of the reporter gene (for example, green fluorescent protein, luciferase, β-galactosidase or chloramphenicol acetyl transferase) is detected when placed under the control of a biologically active polynucleotide fragment. Genomic sequences located upstream of the first exon of the gene may be cloned into any suitable promoter reporter vector. For example, a number of commercially available vectors can be engineered to insert the super-PSA regulatory region of the invention for expression in mammalian host cells. Non-limiting examples of such vectors are pSEAPBasic, pSEAP-Enhancer, pβgal-Basic, pβgal-Enhancer, or pEGFP-1 Promoter Reporter vectors (Clontech, Palo Alto, CA) or pGL2-basic or pGL3-basic promoterless luciferase reporter gene vector (Promega, Madison, WI). Each of these promoter reporter vectors include multiple cloning sites positioned upstream of a reporter gene encoding a readily assayable protein such as

secreted alkaline phosphatase, green fluorescent protein, luciferase or β-galactosidase. The regulatory sequences of the PSA gene are inserted into the cloning sites upstream of the reporter gene in both orientations and introduced into an appropriate host cell. The level of reporter protein is assayed and compared to the level obtained with a vector lacking an insert in the cloning site. The presence of an elevated expression level in the vector containing the insert with respect the control vector indicates the presence of a promoter in the insert.

Expression vectors that comprise a super-PSA regulatory region may further contain a gene encoding a selectable marker. A number of selection systems may be used, including but not limited to, the herpes simplex virus thymidine kinase (Wigler *et al.*, 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, Proc. Natl. Acad. Sci. USA 48:2026) and adenine phosphoribosyltransferase (Lowy *et al.*, 1980, Cell 22:817) genes, which can be employed in tk^-, $hgprt^-$ or $aprt^-$ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for *dhfr*, which confers resistance to methotrexate (Wigler *et al.*, 1980, Proc. Natl. Acad. Sci. USA 77:3567; O'Hare *et al.*, 1981, Proc. Natl. Acad. Sci. USA 78:1527); *gpt*, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, Proc. Natl. Acad. Sci. USA 78:2072); *neo*, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin *et al.*, 1981, J. Mol. Biol. 150:1); and *hygro*, which confers resistance to hygromycin (Santerre *et al.*, 1984, Gene 30:147) genes. Additional selectable genes include *trpB*, which allows cells to utilize indole in place of tryptophan; *hisD*, which allows cells to utilize histinol in place of histidine (Hartman & Mulligan, 1988, Proc. Natl. Acad. Sci. USA 85:8047); *ODC* (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue L., 1987, In: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory ed.) and glutamine synthetase (Bebbington *et al.*, 1992, Biotech 10:169).

1.2.2 Characterization of Transcriptionally Active Regulatory Fragments

A fusion construct comprising a super-PSA regulatory region, or a fragment thereof, can be assayed for transcriptional activity. As a first step in promoter analysis, the transcriptional start point (+1 site) of the prostate-specific gene under study has to be determined using primer extension assay and/or RNAase protection assay, following standard methods (Sambrook *et al.*,1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, Cold Spring Harbor Press). The DNA sequence upstream of the +1 site is

generally considered as the promoter region responsible for gene regulation. However, downstream sequences, including sequences within introns, also may be involved in gene regulation. To begin testing for promoter activity, a -3 kb to +3 kb region (where +1 is the transcriptional start point) may be cloned upstream of the reporter gene coding region. Two or more additional reporter gene constructs also may be made which contain 5' and/or 3' truncated versions of the regulatory region to aid in identification of the region responsible for prostate-specific expression. The choice of the type of reporter gene is made based on the application.

In a preferred embodiment, a GFP reporter gene construct is used. The application of green fluorescent protein (GFP) as a reporter is particularly useful in the study of prostate-specific gene promoters. A major advantage of using GFP as a reporter lies in the fact that GFP can be detected in freshly isolated prostate cells without the need for substrates.

In another embodiment of the invention, a *Lac Z* reporter construct is used. The *Lac Z* gene product, β-galactosidase, is extremely stable and has a broad specificity so as to allow the use of different histochemical, chromogenic or fluorogenic substrates, such as, but not limited to, 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-gal), lactose 2,3,5-triphenyl-2H-tetrazolium (lactose-tetrazolium) and fluorescein galactopyranoside (see Nolan *et al.*, 1988, *supra*).

For promoter analysis in transgenic mice, GFP that has been optimized for expression in mammalian cells is preferred. The promoterless cloning vector pEGFP1 (Clontech, Palo Alto, CA) encodes a red shifted variant of the wild-type GFP which has been optimized for brighter fluorescence and higher expression in mammalian cells (Cormack *et al.*, 1996, Gene 173:33; Haas *et al.*, 1996, Curr. Biol. 6: 315). Moreover, since the maximal excitation peak of this enhanced GFP (EGFP) is at 488 nm, commonly used filter sets such as fluorescein isothiocyanate (FITC) optics which illuminate at 450-500 nm can be used to visualize GFP fluorescence. pEGFP1 proved to be useful as a reporter vector for promoter analysis in transgenic mice (Okabe *et al*, 1997, FEBS Lett. 407: 313). In an alternate embodiment, transgenic mice containing transgenes with a super-PSA regulatory region upstream of the *Lac Z* or luciferase reporter genes are utilized.

Putative promoter fragments can be prepared (usually from a parent phage clone containing 8-10 kb genomic DNA including the promoter region) for cloning using methods known in the art. However, the feasibility of this method depends on the availability of proper restriction endonuclease sites in the regulatory fragment. In a preferred embodiment, the required promoter fragment is amplified by polymerase chain reaction (PCR; Saiki *et al.*, 1988, Science 239:487) using oligonucleotide primers bearing

the appropriate sites for restriction endonuclease cleavage. The sequence necessary for restriction cleavage is included at the 5' end of the forward and reverse primers which flank the regulatory fragment to be amplified. After PCR amplification, the appropriate ends are generated by restriction digestion of the PCR product. The promoter fragments, generated by either method, are then ligated into the multiple cloning site of the reporter vector following standard cloning procedures (Sambrook *et al.*,1989, *supra*). It is recommended that the DNA sequence of the PCR generated promoter fragments in the constructs be verified prior to generation of transgenic animals. The resulting reporter gene construct will contain the putative promoter fragment located upstream of the reporter gene open reading frame, *e.g.*, GFP or *Lac Z* cDNA.

In the preferred embodiment, the following protocol is used. Fifty to 100 pg of the reporter gene construct is digested using appropriate restriction endonucleases to release the transgene fragment. The restriction endonuclease cleaved products are resolved in a 1% (w/v) agarose gel containing 0.5 ug/ml ethidium bromide and TAE buffer (lX: 0.04 M Tri-acetate, 0.001 M EDTA, pH 8.0) at 5-6 V/cm. The transgene band is located by size using a UV transilluminator, preferably using long-wavelength UV lamp to reduce nicking of DNA, and the gel piece containing the required band carefully excised. The gel slice and 1 ml of 0.5 X TAE buffer is added to a dialysis bag, which has been boiled in 1 mM EDTA, pH 8.0 for 10 minutes (Sambrook *et al.*,1989, *supra*) and the ends are fastened. The dialysis bag containing the gel piece is submerged in a horizontal gel electrophoresis chamber containing 0.5 X TAE buffer, and electrophoresed at 5-6 V/cm for 45 minutes. The current flow in the electrophoresis chamber is reversed for one minute before stopping the run to release the DNA which may be attached to the wall of the dialysis tube. The TAE buffer containing the electroeluted DNA from the dialysis bag is collected in a fresh eppendorf tube. The gel piece may be observed on the UV transilluminator to ascertain that the electroelution of the DNA is complete.

The electroeluted DNA sample is further purified by passing through Elutip D columns. The matrix of the column is prewashed with 1-2 ml of High salt buffer (1.0 M NaCl, 20mM Tris. Cl, 1.0 mM EDTA, pH 7.5), followed by a wash with 5 ml of Low salt buffer (0.2 M NaCl, 20 mM Tris. Cl, 1.0 mM EDTA, pH 7.5). A 5 ml syringe is used to apply solutions to the Elutip D column, avoiding reverse flow. The solution containing the electroeluted DNA is loaded slowly. The column is washed with 2-3 ml of Low salt buffer and the DNA is eluted in 0.4 ml of High salt buffer. Two volumes of cold 95% ethanol is added to precipitate DNA. The DNA is collected by centrifugation in a microcentrifuge at 14,000 g for 10 minutes, carefully removing the alcohol without disrupting the DNA pellet. The pellet is washed at least twice with 70% (v/v) ethanol, and dried. The washing and

drying steps are important, as residual salt and ethanol are lethal to the developing embryos. The DNA is resuspend in the injection buffer (10mM TM, 0.1 mM EDTA, pH 7.5 prepared with Milli-Q quality water). The concentration of the purified transgene DNA fragment is determined by measuring the optical density at A_{260} (A_{260} = 1 for 50 µg/ml DNA) using a spectrophotometer. DNA prepared in this manner is suitable for microinjection into fertilized mouse eggs.

1.2.3 Prostate-Specific Promoter Analysis Using Transgenic Mice

The mammalian super-PSA regulatory region can be used to direct expression of, *inter alia*, a reporter coding sequence, a homologous gene or a heterologous gene in transgenic animals. Animals of any species, including, but not limited to, mice, rats, rabbits, guinea pigs, pigs, micro-pigs, goats, sheep, and non-human primates, *e.g.*, baboons, monkeys and chimpanzees may be used to generate transgenic animals. The term "transgenic," as used herein, refers to non-human animals expressing super-PSA sequences from a different species (*e.g.*, mice expressing super-PSA sequences), as well as animals that have been genetically engineered to over-express endogenous (*i.e.*, same species) super-PSA sequences or animals that have been genetically engineered to knock-out specific sequences.

In one embodiment, the present invention provides for transgenic animals that carry a transgene such as a reporter gene under the control of the super-PSA regulatory region or transcriptionally active fragments thereof in all their cells, as well as animals that carry the transgene in some, but not all their cells, *i.e.*, mosaic animals. The transgene may be integrated as a single transgene or in concatamers, *e.g.*, head-to-head tandems or head-to-tail tandems. The transgene may also be selectively introduced into and activated in a particular cell type by following, for example, the teaching of Lasko *et al.* (1992, Proc. Natl. Acad. Sci. USA 89:6232-6236). When it is desired that the transgene be integrated into the chromosomal site of the endogenous corresponding gene, gene targeting is preferred. Briefly, when such a technique is to be utilized, vectors containing some nucleotide sequences homologous to the endogenous gene are designed for the purpose of integrating, via homologous recombination with chromosomal sequences, into and disrupting the function of the nucleotide sequence of the endogenous gene.

Any technique known in the art may be used to introduce a transgene under the control of the super-PSA regulatory region into animals to produce the founder lines of transgenic animals. Such techniques include, but are not limited to, pronuclear microinjection (Hoppe & Wagner, 1989, U.S. Patent No. 4,873,191); nuclear transfer into

enucleated oocytes of nuclei from cultured embryonic, fetal or adult cells induced to quiescence (Campbell *et al.*, 1996, Nature 380:64-66; Wilmut *et al.*, Nature 385:810-813); retrovirus gene transfer into germ lines (Van der Putten *et al.*, 1985, Proc. Natl. Acad. Sci., USA 82:6148-6152); gene targeting in embryonic stem cells (Thompson *et al.*, 1989, Cell 65:313-321); electroporation of embryos (Lo, 1983, Mol. Cell. Biol. 31:1803-1814); and sperm-mediated gene transfer (Lavitrano *et al.*, 1989, Cell 57:717-723; see, Gordon, 1989, Transgenic Animals, Intl. Rev. Cytol. 115:171-229).

For example, for microinjection of fertilized eggs, a linear DNA fragment (the transgene) containing the regulatory region, the reporter gene and the polyadenylation signals, is excised from the reporter gene construct. The transgene may be gel purified by methods known in the art, for example, by the electroelution method. Following electroelution of gel fragments, any traces of impurities are further removed by passing through Elutip D column (Schleicher & Schuell, Dassel, Germany).

1.3 Screening Assays

Compounds that interfere with the abnormal function and/or growth of prostate cells can provide therapies targeting defects in prostate-related disorders. Such compounds may be used to interfere with the onset or the progression of prostate-related disorders. Compounds that stimulate or inhibit promoter activity may be used to ameliorate symptoms of prostate-related disorders.

Transgenic animals or cells containing a super-PSA regulatory region, or fragment thereof, operably linked to a reporter gene, can be used as systems for the screening of agents that modulate super-PSA transcriptional activity. In addition, super-PSA containing transgenic mice provide an experimental model both *in vivo* and *in vitro* to develop new methods of treating prostate-related disorders by targeting drugs to cause arrest in the progression of such disorders.

The present invention encompasses screening assays designed to identify compounds that modulate activity of the super-PSA regulatory region. The present invention encompasses *in vitro* and cell-based assays, as well as *in vivo* assays in transgenic animals. As described hereinbelow, compounds to be tested may include, but are not limited to, oligonucleotides, peptides, proteins, small organic or inorganic compounds, antibodies, *etc.*

Examples of compounds may include, but are not limited to, peptides, such as, for example, soluble peptides, including, but not limited to, Ig-tailed fusion peptides, and members of random peptide libraries; (see, *e.g.*, Lam, *et al.*, 1991, *Nature* 354:82-84;

Houghten, *et al.*, 1991, *Nature* 354:84-86), and combinatorial chemistry-derived molecular library made of D- and/or L- configuration amino acids, phosphopeptides (including, but not limited to members of random or partially degenerate, directed phosphopeptide libraries; see, *e.g.*, Songyang, *et al.*, 1993, *Cell* 72:767-778), antibodies (including, but not limited to, polyclonal, monoclonal, humanized, anti-idiotypic, chimeric or single chain antibodies, and FAb, $F(ab')_2$ and FAb expression library fragments, and epitope-binding fragments thereof), and small organic or inorganic molecules.

Such compounds may further comprise compounds, in particular drugs or members of classes or families of drugs, known to ameliorate the symptoms of a prostate-related disorder.

Such compounds include, but are not limited to, families of antidepressants such as lithium salts, carbamazepine, valproic acid, lysergic acid diethylamide (LSD), *p*-chlorophenylalanine, *p*-propyldopacetamide dithiocarbamate derivatives *e.g.*, FLA 63; anti-anxiety drugs, *e.g.*, diazepam; monoamine oxidase (MAO) inhibitors, *e.g.*, iproniazid, clorgyline, phenelzine and isocarboxazid; biogenic amine uptake blockers, *e.g.*, tricyclic antidepressants such as desipramine, imipramine and amitriptyline; serotonin reuptake inhibitors *e.g.*, fluoxetine; antipsychotic drugs such as phenothiazine derivatives (*e.g.*, chlorpromazine (thorazine) and trifluopromazine)), butyrophenones (*e.g.*, haloperidol (Haldol)), thioxanthene derivatives (*e.g.*, chlorprothixene), and dibenzodiazepines (*e.g.*, clozapine); benzodiazepines; dopaminergic agonists and antagonists *e.g.*, L-DOPA, cocaine, amphetamine, α-methyl-tyrosine, reserpine, tetrabenazine, benzotropine, pargyline; noradrenergic agonists and antagonists *e.g.*, clonidine, phenoxybenzamine, phentolamine, tropolone; nitrovasodilators (*e.g.*, nitroglycerine, nitroprusside as well as NO synthase enzymes); and growth factors (*e.g.*, VEGF, FGF, angiopoetins and endostatin).

In one preferred embodiment, primary cultures of germ cells containing a mammalian super-PSA regulatory region operatively linked to a heterologous gene are used to develop assay systems to screen for compounds which can inhibit sequence-specific DNA-protein interactions. Such methods comprise contacting a compound to a cell that expresses a gene under the control of a super-PSA regulatory region, or a transcriptionally active fragment thereof, measuring the level of the gene expression or gene product activity and comparing this level to the level of gene expression or gene product activity produced by the cell in the absence of the compound, such that if the level obtained in the presence of the compound differs from that obtained in its absence, a compound capable of modulating the expression of the mammalian super-PSA regulatory region has been identified. Alterations in gene expression levels may be by any number of methods known to those of skill in the art *e.g.*, by assaying for reporter gene activity, assaying cell lysates for mRNA

transcripts, *e.g.* by Northern analysis or using other methods known in the art for assaying for gene products expressed by the cell.

In another embodiment, microdissection and transillumination can be used. These techniques offer a rapid assay for monitoring effects of putative drugs on prostate cells in transgenic animals containing a super-PSA regulatory region-driven reporter gene. In this embodiment, a test agent is delivered to the transgenic animal by any of a variety of methods. Methods of introducing a test agent may include oral, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal and via scarification (scratching through the top layers of skin, *e.g.*, using a bifurcated needle) or any other standard routes of drug delivery. The effect of such test compounds on the prostate cells can be analyzed by the microdissection and transillumination of the prostate cells. If the level of reporter gene expression observed or measured in the presence of the compound differs from that obtained in its absence, a compound capable of modulating the expression of the mammalian super-PSA regulatory region has been identified.

In various embodiments of the invention, compounds that may be used in screens for modulators of prostate-related disorders include peptides, small molecules, both naturally occurring and/or synthetic (*e.g.*, libraries of small molecules or peptides), cell-bound or soluble molecules, organic, non-protein molecules and recombinant molecules that may have super-PSA regulatory region binding capacity and, therefore, may be candidates for pharmaceutical agents.

Alternatively, the proteins and compounds include endogenous cellular components which interact with super-PSA regulatory region sequences *in vivo*. Cell lysates or tissue homogenates may be screened for proteins or other compounds which bind to the super-PSA regulatory region, or fragment thereof. Such endogenous components may provide new targets for pharmaceutical and therapeutic interventions.

In one embodiment, libraries can be screened. Many libraries are known in the art that can be used, *e.g.*, peptide libraries, chemically synthesized libraries, recombinant (*e.g.*, phage display libraries), and *in vitro* translation-based libraries. In one embodiment of the present invention, peptide libraries may be used to screen for agonists or antagonists of super-PSA-linked reporter expression. Diversity libraries, such as random or combinatorial peptide or non-peptide libraries can be screened for molecules that specifically modulate super-PSA regulatory region activity. Random peptide libraries consisting of all possible combinations of amino acids attached to a solid phase support may be used to identify peptides that are able to activate or inhibit super-PSA regulatory region activities (Lam, K.S. *et al.*, 1991, Nature 354: 82-84). The screening of peptide libraries may have therapeutic value in the discovery of pharmaceutical agents that stimulate or

inhibit the expression of super-PSA by interaction with the promoter region.

Examples of chemically synthesized libraries are described in Fodor *et al.*, 1991, Science 251:767-773; Houghten *et al.*, 1991, Nature 354:84-86; Lam *et al.*, 1991, Nature 354:82-84; Medynski, 1994, BioTechnology 12:709-710; Gallop *et al.*, 1994, J. Medicinal Chemistry 37(9):1233-1251; Ohlmeyer *et al.*, 1993, Proc. Natl. Acad. Sci. USA 90:10922-10926; Erb *et al.*, 1994, Proc. Natl. Acad. Sci. USA 91:11422-11426; Houghten *et al.*, 1992, Biotechniques 13:412; Jayawickreme *et al.*, 1994, Proc. Natl. Acad. Sci. USA 91:1614-1618; Salmon *et al.*, 1993, Proc. Natl. Acad. Sci. USA 90:11708-11712; PCT Publication No. WO 93/20242; and Brenner and Lerner, 1992, Proc. Natl. Acad. Sci. USA 89:5381-5383.

Examples of phage display libraries are described in Scott and Smith, 1990, Science 249:386-390; Devlin *et al.*, 1990, Science, 249:404-406; Christian, *et al.*, 1992, J. Mol. Biol. 227:711-718; Lenstra, 1992, J. Immunol. Meth. 152:149-157; Kay *et al.*, 1993, Gene 128:59-65; and PCT Publication No. WO 94/18318 dated August 18, 1994.

By way of example of non-peptide libraries, a benzodiazepine library (*see e.g.*, Bunin *et al.*, 1994, Proc. Natl. Acad. Sci. USA 91:4708-4712) can be adapted for use. Peptoid libraries (Simon *et al.*, 1992, Proc. Natl. Acad. Sci. USA 89:9367-9371) also can be used. Another example of a library that can be used, in which the amide functionalities in peptides have been permethylated to generate a chemically transformed combinatorial library, is described by Ostresh *et al.* (1994, Proc. Natl. Acad. Sci. USA 91:11138-11142).

A specific embodiment of such an *in vitro* screening assay is described below. The super-PSA regulatory region-reporter vector is used to generate transgenic mice from which primary cultures of super-PSA regulatory region-reporter vector germ cells are established. About 10,000 cells per well are plated in 96-well plates in total volume of 100 µl, using medium appropriate for the cell line. Candidate inhibitors of PSA gene expression are added to the cells. The effect of the inhibitors of PSA gene activation can be determined by measuring the response of the reporter gene driven by the super-PSA regulatory region. This assay could easily be set up in a high-throughput screening mode for evaluation of compound libraries in a 96-well format that reduce (or increase) reporter gene activity, but which are not cytotoxic. After 6 hours of incubation, 100 µl DMEM medium + 2.5% fetal bovine serum (FBS) to 1.25% final serum concentration is added to the cells, which are incubated for a total of 24 hours (18 hours more). At 24 hours, the plates are washed with PBS, blot dried, and frozen at –80°C. The plates are thawed the next day and analyzed for the presence of reporter activity.

In a preferred example of an *in vivo* screening assay, prostate cells derived from transgenic mice can be transplanted into mice with a normal or other desired

phenotype (Brinster *et al.*, 1994, Proc. Natl. Acad. Sci. USA 91: 11298-302; Ogawa *et al.*, 1997, Int. J. Dev. Biol. 41:111-12). Such mice can then be used to test the effect of compounds and other various factors on prostate-related disorders. In addition to the compounds and agents listed above, such mice can be used to assay factors or conditions that can be difficult to test using other methods, such as dietary effects, internal pH, temperature, *etc.*

Once a compound has been identified that inhibits or enhances super-PSA regulatory region activity, it may then be tested in an animal-based assay to determine if the compound exhibits the ability to act as a drug to ameliorate and/or prevent symptoms of a prostate-related disorder, including, but not limited to, prostate cancer.

The assays of the present invention may be first optimized on a small scale (*i.e.*, in test tubes), and then scaled up for high-throughput assays. The screening assays of the present invention may be performed *in vitro*, *i.e.*, in test tubes, using purified components or cell lysates. The screening assays of the present invention may also be carried out in intact cells in culture and in animal models. In accordance with the present invention, test compounds which are shown to modulate the activity of the super-PSA regulatory region *in vitro*, as described herein, will further be assayed *in vivo* in cultured cells and animal models to determine if the test compound has the similar effects *in vivo* and to determine the effects of the test compound on prostate-related disorders.

1.4 Compositions and Methods for Therapeutic Use of Super-PSA Nucleotides

Super-PSA polynucleotides, or transcriptionally active fragments thereof, can be used to treat and/or prevent diseases, conditions or disorders that can be ameliorated by modifying the level or the expression of PSA, or a heterologous gene linked to a super-PSA regulatory region, in an prostate-specific manner. Described herein are methods for such therapeutic treatments.

The super-PSA regulatory region may be used to achieve tissue specific expression in gene therapy protocols. In cases where such cells are tumor cells, the induction of a cytotoxic product by the super-PSA regulatory region may be used in the form of cancer gene therapy specifically targeted to prostate tumor cells which contain trans-acting factors required for PSA expression. In this way, the super-PSA regulatory region may serve as a delivery route for a gene therapy approach to cancers involving the prostate. Additionally, antisense, antigene or aptameric oligonucleotides may be delivered to cells using the presently described expression constructs. Ribozymes or single-stranded

RNA also can be expressed in a cell to inhibit the expression of a target gene of interest. The target genes for these antisense or ribozyme molecules should be those encoding gene products that are essential for cell maintenance.

The super-PSA regulatory region, and transcriptionally active fragments thereof, of the present invention may be used for a wide variety of purposes, *e.g.*, to down regulate PSA gene expression, or, alternatively, to achieve prostate-specific, stage-specific expression of heterologous genes.

In one embodiment, for example, the endogenous super-PSA regulatory region may be targeted to specifically down-regulate expression of the PSA gene. For example, oligonucleotides complementary to the regulatory region may be designed and delivered to the cells. Such oligonucleotides may anneal to the regulatory sequence and prevent transcription activation. Alternatively, the regulatory sequence, or portions thereof, may be delivered to cells in saturating concentrations to compete for transcription factor binding. For general reviews of the methods of gene therapy, see Goldspiel *et al.*, 1993, Clinical Pharmacy 12:488-505; Wu and Wu, 1991, Biotherapy 3:87-95; Tolstoshev, 1993, Ann. Rev. Pharmacol. Toxicol. 32:573-596; Mulligan, 1993, Science 260:926-932; and Morgan and Anderson, 1993, Ann. Rev. Biochem. 62:191-217; May, 1993, TIBTECH 11:155-215. Methods commonly known in the art of recombinant DNA technology which can be used are described in Ausubel *et al.* (eds.), 1993, Current Protocols in Molecular Biology, John Wiley & Sons, NY; and Kriegler, 1990, Gene Transfer and Expression, A Laboratory Manual, Stockton Press, NY.

In another embodiment, a gene therapy method for ameliorating prostate-related disorders is provided. Super-PSA regulatory region sequences are introduced in the prostate and used to drive prostate-specific expression of drugs or toxins. The method comprises introducing a super-PSA regulatory region sequence operatively associated with a drug or toxin gene into the prostate.

In yet another embodiment, the invention provides a gene therapy method for treatment of cancer or other proliferative disorders. The super-PSA regulatory region is used to direct the expression of one or more proteins specifically in prostate tumor cells of a patient. Such proteins may be, for example, tumor suppressor genes, thymidine kinase (used in combination with acyclovir), toxins or proteins involved in cell killing, such as proteins involved in the apoptosis pathway.

In still another embodiment, the invention provides a preventative gene therapy method for preventing and/or delaying the onset of prostate-related disorders. The super-PSA regulatory region is introduced in the prostate and used to drive prostate-specific expression of therapeutic compounds. The method comprises introducing a super-PSA

regulatory region sequence operatively associated with a nucleic acid encoding a therapeutic compound into the prostate to prevent and/or delay the onset of prostate-related disorders.

Methods for introducing genes for expression in mammalian cells are well known in the field. Generally, for such gene therapy methods, the nucleic acid is directly administered *in vivo* into a target cell or a transgenic mouse that expresses a super-PSA regulatory region operably linked to a reporter gene. This can be accomplished by any method known in the art, *e.g.*, by constructing it as part of an appropriate nucleic acid expression vector and administering it so that it becomes intracellular, *e.g.*, by infection using a defective or attenuated retroviral or other viral vector (see U.S. Patent No. 4,980,286), by direct injection of naked DNA, by use of microparticle bombardment (*e.g.*, a gene gun; Biolistic, Dupont), by coating with lipids or cell-surface receptors or transfecting agents, by encapsulation in liposomes, microparticles, or microcapsules, by administering it in linkage to a peptide which is known to enter the nucleus or by administering it in linkage to a ligand subject to receptor-mediated endocytosis (see e.g., Wu and Wu, 1987, J. Biol. Chem. 262:4429-4432), which can be used to target cell types specifically expressing the receptors. In another embodiment, a nucleic acid-ligand complex can be formed in which the ligand comprises a fusogenic viral peptide to disrupt endosomes, allowing the nucleic acid to avoid lysosomal degradation. In yet another embodiment, the nucleic acid can be targeted *in vivo* for cell specific uptake and expression, by targeting a specific receptor (see, e.g., PCT Publications WO 92/06180 dated April 16, 1992; WO 92/22635 dated December 23, 1992; WO92/20316 dated November 26, 1992; WO93/14188 dated July 22, 1993; WO 93/20221 dated October 14, 1993). Alternatively, the nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination (Koller and Smithies, 1989, Proc. Natl. Acad. Sci. USA 86:8932-8935; Zijlstra *et al.*, 1989, Nature 342:435-438).

The oligonucleotide may comprise at least one modified base moiety which is selected from the group including, but not limited to: 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5′-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-

5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine.

Endogenous target gene expression also can be reduced by inactivating or "knocking out" the super-PSA regulatory region using targeted homologous recombination (*e.g.*, see Smithies *et al.*, 1985, Nature 317:230-234; Thomas and Capecchi, 1987, Cell 51:503-512; Thompson *et al.*, 1989, Cell 5:313-321; each of which is incorporated by reference herein in its entirety). For example, a mutant, non-functional target gene (or a completely unrelated DNA sequence) flanked by DNA homologous to the regulatory region of the PSA gene can be used, with or without a selectable marker and/or a negative selectable marker, to transfect cells that express the target gene *in vivo*. Insertion of the DNA construct, via targeted homologous recombination, results in inactivation of the super-PSA regulatory region. This approach can be adapted for use in humans provided the recombinant DNA constructs are directly administered or targeted to the required site *in vivo* using appropriate vectors.

In an alternative embodiment, endogenous target gene expression can be reduced by targeting deoxyribonucleotide sequences complementary to the super-PSA regulatory region to form triple helical structures that prevent transcription of the target gene in target cells in the body. (See generally, Helene, 1991, Anticancer Drug Des., 6(6):569-584; Helene *et al.*, 1992, Ann. N.Y. Acad. Sci., 660:27-36; and Maher, 1992, Bioassays 14(12):807-815).

Nucleic acid molecules to be used in triple helix formation for the inhibition of transcription should be single stranded and composed of deoxynucleotides. The base composition of these oligonucleotides must be designed to promote triple helix formation via Hoogsteen base pairing rules, which generally require sizeable stretches of either purines or pyrimidines to be present on one strand of a duplex. Nucleotide sequences may be pyrimidine-based, which will result in TAT and CGC triplets across the three associated strands of the resulting triple helix. The pyrimidine-rich molecules provide base complementarity to a purine-rich region of a single strand of the duplex in a parallel orientation to that strand. In addition, nucleic acid molecules may be chosen that are purine-rich, for example, contain a stretch of G residues. These molecules will form a triple helix with a DNA duplex that is rich in GC pairs, in which the majority of the purine residues are located on a single strand of the targeted duplex, resulting in GGC triplets across the three strands in the triplex.

In a specific embodiment, the potential sequences that can be targeted for triple helix formation may be increased by creating a so called "switchback" nucleic acid molecule. Switchback molecules are synthesized in an alternating 5'-3', 3'-5' manner, such

that they base pair with first one strand of a duplex and then the other, eliminating the necessity for a sizeable stretch of either purines or pyrimidines to be present on one strand of a duplex.

The anti-sense RNA and DNA molecules and triple helix molecules of the invention may be prepared by any method known in the art for the synthesis of nucleic acid molecules. These include techniques for chemically synthesizing oligodeoxyribonucleotides well known in the art such as for example solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by *in vitro* and *in vivo* transcription of DNA sequences encoding the RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors which contain suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines.

Various modifications to the DNA molecules may be introduced as a means of increasing intracellular stability and half-life. Possible modifications include, but are not limited to, the addition of flanking sequences of ribo- or deoxy- nucleotides to the 5' and/or 3' ends of the molecule or the use of phosphorothioate or 2' O-methyl rather than phosphodiesterase linkages within the oligodeoxyribonucleotide backbone.

The super-PSA regulatory region, and transcriptionally active fragments thereof, of the present invention can be used to express the PSA gene in an altered manner as compared to expression in a normal cell. The super-PSA regulatory region, and transcriptionally active fragments thereof, of the present invention also can be used to achieve tissue specific expression of a target gene. Thus, it is possible to design appropriate therapeutic and diagnostic techniques directed to this regulatory sequence in order to modulate the expression of a target gene. In accordance with the present invention, the term "modulate" encompasses the suppression or augmentation of expression of a target gene and also encompasses the tissue specific suppression or expression of a target gene. When a cell proliferative disorder is associated with underexpression or overexpression of a PSA gene product, oligonucleotide based compounds such as those described herein, including antisense oligonucleotides, may be used to modulate expression of the PSA gene. For example, where the associated disorder is cancer, the induction of a cytotoxic gene product utilizing the super-PSA regulatory region may be used as a cancer therapy. One of skill in the art can determine if a particular therapeutic course of treatment is successful by several methods known to those of skill in the art, including muscle fiber analysis or biopsy.

1.4.1 Inhibitory, Antisense, Ribozyme and Triple Helix Approaches

In another embodiment, symptoms of disorders involving the prostate may be ameliorated by decreasing the level of super-PSA regulatory region activity by using well-known antisense, gene "knock-out," ribozyme and/or triple helix methods to decrease the level of super-PSA regulatory region expression. Among the compounds that exhibit the ability to modulate the activity, expression or synthesis of the super-PSA regulatory region, including the ability to ameliorate the symptoms of a prostate-related disorder are antisense, ribozyme and triple helix molecules. Such molecules may be designed to reduce or inhibit either unimpaired, or if appropriate, mutant super-PSA regulatory region activity. Techniques for the production and use of such molecules are well known to those of skill in the art.

Antisense RNA and DNA molecules act to directly block the translation of mRNA by hybridizing to targeted mRNA and preventing protein translation. Antisense approaches involve the design of oligonucleotides that are complementary to a target gene mRNA. The antisense oligonucleotides will bind to the complementary target gene mRNA transcripts and prevent translation. Absolute complementarity, although preferred, is not required.

A sequence "complementary" to a portion of an RNA, as referred to herein, means a sequence having sufficient complementarity to be able to hybridize with the RNA, forming a stable duplex; in the case of double-stranded antisense nucleic acids, a single strand of the duplex DNA may thus be tested, or triplex formation may be assayed. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches with an RNA it may contain and still form a stable duplex (or triplex, as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

In one embodiment, oligonucleotides complementary to non-coding regions of the gene of interest could be used in an antisense approach to inhibit translation of endogenous mRNA. Antisense nucleic acids should be at least six nucleotides in length, and are preferably oligonucleotides ranging from 6 to about 50 nucleotides in length. In specific aspects, the oligonucleotide is at least 10 nucleotides, at least 17 nucleotides, at least 25 nucleotides or at least 50 nucleotides.

Regardless of the choice of target sequence, it is preferred that *in vitro* studies are first performed to quantitate the ability of the antisense oligonucleotide to inhibit target gene expression. It is preferred that these studies utilize controls that distinguish

between antisense gene inhibition and nonspecific biological effects of oligonucleotides. It is also preferred that these studies compare levels of the target RNA or protein with that of an internal control RNA or protein. Additionally, it is envisioned that results obtained using the antisense oligonucleotide are compared with those obtained using a control oligonucleotide. It is preferred that the control oligonucleotide is of approximately the same length as the test oligonucleotide and that the nucleotide sequence of the oligonucleotide differs from the antisense sequence no more than is necessary to prevent specific hybridization to the target sequence.

The oligonucleotides can be DNA or RNA or chimeric mixtures or derivatives or modified versions thereof, single-stranded or double-stranded. The oligonucleotide can be modified at the base moiety, sugar moiety, or phosphate backbone, for example, to improve stability of the molecule, hybridization, etc. The oligonucleotide may include other appended groups such as peptides (*e.g.*, for targeting host cell receptors *in vivo*), or agents facilitating transport across the cell membrane (see, *e.g.*, Letsinger, *et al.*, 1989, *Proc. Natl. Acad. Sci. U.S.A.* 86:6553-6556; Lemaitre, *et al.*, 1987, *Proc. Natl. Acad. Sci. U.S.A.* 84:648-652; PCT Publication No. WO88/09810, published December 15, 1988) or the blood-brain barrier (see, *e.g.*, PCT Publication No. WO89/10134, published April 25, 1988), hybridization-triggered cleavage agents (see, *e.g.*, Krol *et al.*, 1988, *BioTechniques* 6:958-976) or intercalating agents (see, *e.g.*, Zon, 1988, *Pharm. Res.* 5:539-549). To this end, the oligonucleotide may be conjugated to another molecule, *e.g.*, a peptide, hybridization triggered cross-linking agent, transport agent, hybridization-triggered cleavage agent, *etc.*

The antisense oligonucleotide may comprise at least one modified base moiety which is selected from the group including but not limited to 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5′-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine.

The antisense oligonucleotide may also comprise at least one modified sugar

moiety selected from the group including but not limited to arabinose, 2-fluoroarabinose, xylulose, and hexose.

In yet another embodiment, the antisense oligonucleotide comprises at least one modified phosphate backbone selected from the group consisting of a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

In yet another embodiment, the antisense oligonucleotide is an α-anomeric oligonucleotide. An α-anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β-units, the strands run parallel to each other (Gautier, *et al.*, 1987, *Nucl. Acids Res.* 15:6625-6641). The oligonucleotide is a 2'-0-methylribonucleotide (Inoue, *et al.*, 1987, *Nucl. Acids Res.* 15:6131-6148), or a chimeric RNA-DNA analogue (Inoue, *et al.*, 1987, *FEBS Lett.* 215:327-330).

Oligonucleotides of the invention may be synthesized by standard methods known in the art, *e.g.*, by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, etc.). As examples, phosphorothioate oligonucleotides may be synthesized by the method of Stein, *et al.* (1988, *Nucl. Acids Res.* 16:3209), methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin, *et al.*, 1988, *Proc. Natl. Acad. Sci. U.S.A.* 85:7448-7451), *etc.*

While antisense nucleotides complementary to the target gene coding region sequence could be used, those complementary to the transcribed, untranslated region are most preferred.

Antisense molecules should be delivered to cells that express the target gene *in vivo*. A number of methods have been developed for delivering antisense DNA or RNA to cells; *e.g.*, antisense molecules can be injected directly into the tissue site, or modified antisense molecules, designed to target the desired cells (*e.g.*, antisense linked to peptides or antibodies that specifically bind receptors or antigens expressed on the target cell surface) can be administered systemically.

A preferred approach to achieve intracellular concentrations of the antisense sufficient to suppress translation of endogenous mRNAs utilizes a recombinant DNA construct in which the antisense oligonucleotide is placed under the control of a strong pol III or pol II promoter. The use of such a construct to transfect target cells in the patient will result in the transcription of sufficient amounts of single stranded RNAs that will form complementary base pairs with the endogenous target gene transcripts and thereby prevent translation of the target gene mRNA. For example, a vector can be introduced *e.g.*, such that it is taken up by a cell and directs the transcription of an antisense RNA. Such a vector

can remain episomal or become chromosomally integrated, as long as it can be transcribed to produce the desired antisense RNA. Such vectors can be constructed by recombinant DNA technology methods standard in the art. Vectors can be plasmid, viral, or others known in the art, used for replication and expression in mammalian cells. Expression of the sequence encoding the antisense RNA can be by any promoter known in the art to act in mammalian, preferably human cells. Such promoters can be inducible or constitutive. Such promoters include but are not limited to: the SV40 early promoter region (Bernoist and Chambon, 1981, *Nature* 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, *et al.*, 1980, *Cell* 22:787-797), the herpes thymidine kinase promoter (Wagner, *et al.*, 1981, *Proc. Natl. Acad. Sci. U.S.A.* 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster, *et al.*, 1982, *Nature* 296:39-42), etc. Any type of plasmid, cosmid, YAC or viral vector can be used to prepare the recombinant DNA construct which can be introduced directly into the tissue site. Alternatively, viral vectors can be used that selectively infect the desired tissue, in which case administration may be accomplished by another route (*e.g.*, systemically).

Ribozyme molecules designed to catalytically cleave target gene mRNA transcripts can also be used to prevent translation of target gene mRNA and, therefore, expression of target gene product. (See, *e.g.*, PCT International Publication WO90/11364, published October 4, 1990; Sarver, *et al.*, 1990, *Science* 247, 1222-1225).

Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. (For a review, see Rossi, 1994, *Current Biology* 4:469-471). The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by an endonucleolytic cleavage event. The composition of ribozyme molecules must include one or more sequences complementary to the target gene mRNA, and must include the well known catalytic sequence responsible for mRNA cleavage. For this sequence, see, *e.g.*, U.S. Patent No. 5,093,246, which is incorporated herein by reference in its entirety.

While ribozymes that cleave mRNA at site specific recognition sequences can be used to destroy target gene mRNAs, the use of hammerhead ribozymes is preferred. Hammerhead ribozymes cleave mRNAs at locations dictated by flanking regions that form complementary base pairs with the target mRNA. The sole requirement is that the target mRNA have the following sequence of two bases: 5'-UG-3'. The construction and production of hammerhead ribozymes is well known in the art and is described more fully in Myers, 1995, *Molecular Biology and Biotechnology: A Comprehensive Desk Reference*, VCH Publishers, New York, (see especially FIG. 4, page 833) and in Haseloff and Gerlach, 1988, *Nature*, 334:585-591, which is incorporated herein by reference in its entirety.

Preferably the ribozyme is engineered so that the cleavage recognition site is located near the 5' end of the target gene mRNA, *i.e.*, to increase efficiency and minimize the intracellular accumulation of non-functional mRNA transcripts.

The ribozymes of the present invention also include RNA endoribonucleases (hereinafter "Cech-type ribozymes") such as the one that occurs naturally in *Tetrahymena thermophila* (known as the IVS, or L-19 IVS RNA) and that has been extensively described by Thomas Cech and collaborators (Zaug, *et al.*, 1984, *Science*, 224:574-578; Zaug and Cech, 1986, *Science*, 231:470-475; Zaug, *et al.*, 1986, *Nature*, 324:429-433; published International patent application No. WO 88/04300 by University Patents Inc.; Been and Cech, 1986, *Cell*, 47:207-216). The Cech-type ribozymes have an eight base pair active site which hybridizes to a target RNA sequence whereafter cleavage of the target RNA takes place. The invention encompasses those Cech-type ribozymes which target eight base-pair active site sequences that are present in the target gene.

As in the antisense approach, the ribozymes can be composed of modified oligonucleotides (*e.g.*, for improved stability, targeting, *etc.*) and should be delivered to cells that express the target gene *in vivo*. A preferred method of delivery involves using a DNA construct "encoding" the ribozyme under the control of a strong constitutive pol III or pol II promoter, so that transfected cells will produce sufficient quantities of the ribozyme to destroy endogenous target gene messages and inhibit translation. Because ribozymes unlike antisense molecules, are catalytic, a lower intracellular concentration is required for efficiency.

Endogenous target gene expression can also be reduced by inactivating or "knocking out" the target gene or its promoter using targeted homologous recombination (*e.g.*, see Smithies, *et al.*, 1985, *Nature* 317:230-234; Thomas and Capecchi, 1987, *Cell* 51:503-512; Thompson, *et al.*, 1989, *Cell* 5:313-321; each of which is incorporated by reference herein in its entirety). For example, a mutant, non-functional target gene (or a completely unrelated DNA sequence) flanked by DNA homologous to the endogenous target gene (either the coding regions or regulatory regions of the target gene) can be used, with or without a selectable marker and/or a negative selectable marker, to transfect cells that express the target gene *in vivo*. Insertion of the DNA construct, via targeted homologous recombination, results in inactivation of the target gene. Such approaches are particularly suited in the agricultural field where modifications to ES (embryonic stem) cells can be used to generate animal offspring with an inactive target gene (*e.g.*, see Thomas and Capecchi, 1987 and Thompson, 1989, *supra*). However this approach can be adapted for use in humans provided the recombinant DNA constructs are directly administered or targeted to the required site *in vivo* using appropriate viral vectors.

Alternatively, endogenous target gene expression can be reduced by targeting deoxyribonucleotide sequences complementary to the regulatory region of the target gene (*i.e.*, the target gene promoter and/or enhancers) to form triple helical structures that prevent transcription of the target gene in target cells in the body. (See generally, Helene, 1991, *Anticancer Drug Des.*, 6(6):569-584; Helene, *et al.*, 1992, *Ann. N.Y. Acad. Sci.*, 660:27-36; and Maher, 1992, *Bioassays* 14(12):807-815).

Nucleic acid molecules to be used in triplex helix formation for the inhibition of transcription should be single stranded and composed of deoxynucleotides. The base composition of these oligonucleotides must be designed to promote triple helix formation via Hoogsteen base pairing rules, which generally require sizeable stretches of either purines or pyrimidines to be present on one strand of a duplex. Nucleotide sequences may be pyrimidine-based, which will result in TAT and CGC^+ triplets across the three associated strands of the resulting triple helix. The pyrimidine-rich molecules provide base complementarity to a purine-rich region of a single strand of the duplex in a parallel orientation to that strand. In addition, nucleic acid molecules may be chosen that are purine-rich, for example, contain a stretch of G residues. These molecules will form a triple helix with a DNA duplex that is rich in GC pairs, in which the majority of the purine residues are located on a single strand of the targeted duplex, resulting in GGC triplets across the three strands in the triplex.

Alternatively, the potential sequences that can be targeted for triple helix formation may be increased by creating a so called "switchback" nucleic acid molecule. Switchback molecules are synthesized in an alternating 5'-3', 3'-5' manner, such that they base pair with first one strand of a duplex and then the other, eliminating the necessity for a sizeable stretch of either purines or pyrimidines to be present on one strand of a duplex.

In instances wherein the antisense, ribozyme, and/or triple helix molecules described herein are utilized to inhibit mutant gene expression, it is possible that the technique may so efficiently reduce or inhibit the transcription (triple helix) and/or translation (antisense, ribozyme) of mRNA produced by normal target gene alleles that the possibility may arise wherein the concentration of normal target gene product present may be lower than is necessary for a normal phenotype. In such cases, to ensure that substantially normal levels of target gene activity are maintained, therefore, nucleic acid molecules that encode and express target gene polypeptides exhibiting normal target gene activity may, be introduced into cells via gene therapy methods, such as those described below, that do not contain sequences susceptible to whatever antisense, ribozyme, or triple helix treatments are being utilized. Alternatively, in instances whereby the target gene encodes an extracellular protein, it may be preferable to co-administer normal target gene

protein in order to maintain the requisite level of target gene activity.

Anti-sense RNA and DNA, ribozyme and triple helix molecules of the invention may be prepared by any method known in the art for the synthesis of DNA and RNA molecules, as discussed above. These include techniques for chemically synthesizing oligodeoxyribonucleotides and oligoribonucleotides well known in the art such as for example solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by *in vitro* and *in vivo* transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors that incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines.

1.4.2 Gene Replacement Therapy

The nucleic acid sequences of the invention, described above, can be utilized for transferring recombinant nucleic acid sequences to cells and expressing said sequences in recipient cells. Such techniques can be used, for example, in marking cells or for the treatment of a prostate-related disorders. Such treatment can be in the form of gene replacement therapy. Specifically, one or more copies of a normal gene or a portion of the gene that directs the production of a gene product exhibiting normal gene function, may be inserted into the appropriate cells within a patient, using vectors that include, but are not limited to adenovirus, adeno-associated virus and retrovirus vectors, in addition to other particles that introduce DNA into cells, such as liposomes.

In one embodiment, techniques for delivery involve direct administration, *e.g.*, by stereotactic delivery of such gene sequences to the site of the cells in which the gene sequences are to be expressed.

Additional methods that may be utilized to increase the overall level of gene expression and/or gene product activity include using targeted homologous recombination methods, as discussed above, to modify the expression characteristics of an endogenous gene in a cell or microorganism by inserting a heterologous DNA regulatory element such that the inserted regulatory element is operatively linked with the endogenous gene in question. Targeted homologous recombination can thus be used to activate transcription of an endogenous gene that is "transcriptionally silent", *i.e.*, is not normally expressed or is normally expressed at very low levels, or to enhance the expression of an endogenous gene that is normally expressed.

Further, the overall level of target gene expression and/or gene product activity may be increased by the introduction of appropriate target gene-expressing cells, preferably autologous cells, into a patient at positions and in numbers that are sufficient to ameliorate the symptoms of a prostate-related disorder. Such cells may be either recombinant or non-recombinant.

When the cells to be administered are non-autologous cells, they can be administered using well known techniques that prevent a host immune response against the introduced cells from developing. For example, the cells may be introduced in an encapsulated form which, while allowing for an exchange of components with the immediate extracellular environment, does not allow the introduced cells to be recognized by the host immune system.

Additionally, compounds, such as those identified via techniques such as those described above that are capable of modulating activity of a super-PSA regulatory region can be administered using standard techniques that are well known to those of skill in the art.

1.5 Pharmaceutical Preparations and Methods of Administration

The compounds that are determined to modify super-PSA regulatory region activity or gene product activity can be administered to a patient at therapeutically effective doses to treat or ameliorate a prostate-related disorder. A therapeutically effective dose refers to that amount of the compound sufficient to result in amelioration of symptoms of such a disorder.

1.5.1 Effective Dose

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD_{50}/ED_{50}. Compounds that exhibit large therapeutic indices are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

The data obtained from the cell culture assays and animal studies can be used

in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC_{50} (*i.e.*, the concentration of the test compound that achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

1.5.2 Formulations and Use

Pharmaceutical compositions for use in accordance with the present invention may be formulated in conventional manner using one or more physiologically acceptable carriers or excipients.

Thus, the compounds and their physiologically acceptable salts and solvates may be formulated for administration by inhalation or insufflation (either through the mouth or the nose) or oral, buccal, parenteral or rectal administration.

For oral administration, the pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (*e.g.*, pregelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (*e.g.*, lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (*e.g.*, magnesium stearate, talc or silica); disintegrants (*e.g.*, potato starch or sodium starch glycolate); or wetting agents (*e.g.*, sodium lauryl sulphate). The tablets may be coated by methods well known in the art. Liquid preparations for oral administration may take the form of, for example, solutions, syrups or suspensions, or they may be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (*e.g.*, sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (*e.g.*, lecithin or acacia); non-aqueous vehicles (*e.g.*, almond oil, oily esters, ethyl alcohol or fractionated vegetable oils); and preservatives (*e.g.*, methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts, flavoring, coloring and sweetening agents as appropriate.

Preparations for oral administration may be suitably formulated to give

controlled release of the active compound.

For buccal administration the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebuliser, with the use of a suitable propellant, *e.g.*, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of *e.g.*, gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The compounds may be formulated for parenteral administration by injection, *e.g.*, by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, *e.g.*, in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, *e.g.*, sterile pyrogen-free water, before use.

The compounds may also be formulated in rectal compositions such as suppositories or retention enemas, *e.g.*, containing conventional suppository bases such as cocoa butter or other glycerides.

In certain embodiments, it may be desirable to administer the pharmaceutical compositions of the invention locally to the area in need of treatment. This may be achieved by, for example, and not by way of limitation, local infusion during surgery, topical application, *e.g.*, in conjunction with a wound dressing after surgery, by injection, by means of a catheter, by means of a suppository, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers. In one embodiment, administration can be by direct injection at the site (or former site) of a malignant tumor or neoplastic or pre-neoplastic tissue.

For topical application, the compounds may be combined with a carrier so that an effective dosage is delivered, based on the desired activity.

In addition to the formulations described previously, the compounds also may be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with

suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

The compositions may, if desired, be presented in a pack or dispenser device that may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration.

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention. Indeed, various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

2 **Example: Promoter Analysis Studies**

In this example, promoter analysis studies data are presented in Figure 1. Wild type PSA promoter is depicted as p61, the length of the promoter is ~6Kb, the locations of androgen response elements (ARE) I, II & III are shown within the promoter. And the transcription start site (TATA box) is represented by the arrow. The full length promoter was inserted upstream to a luciferase reporter gene and were transiently transfected into LNCaP and C4-2 cells. Relative luciferase unit (RLU) is obtained by normalizing the luciferase activity with the internal control-CMV/beta-gal. In the absence of androgen stimulation, the native PSA promoter is ~15 fold more active in C4-2 than in LNCaP. Different deletion constructs were generated to identify regions on the promoter that are important for the activation of PSA promoter in the absence of androgen. p61-2 construct contains internal deletion between ARE II & III. This deletion does nor affect the native promoter activity, therefore the deleted region may not be essential for the PSA promoter activity. p61-5 contains terminal deletion, downstream of the ARE III. The activity of this construct drops about 50%. However, as the deletion extend upstream to ARE III, we see a tremendous decline (~85%) in promoter activity. The additional deletion (500bp) in p61-4

obviously causes this significant decrease in promoter activity. Therefore, when the 500bp (AREc) was deleted in the p61-3 construct, it results in a similar activity drops as the p61-4. This strongly suggests that the AREc is essential for the PSA promoter activity in die absence of androgen.

3 Example: Comparison of the Super Psa Promoter (Arec/ptata) Activity with the Native Psa Promoter (P61) and the Pse Promoter (Arec/(Pa8)

A comparison of the super PSA promoter (AREc/pTATA) activity with the native PSA promoter (p61) and the PSE promoter (AREc/(PA8). In addition to AREc, the possible cooperativity between AREI, II and III was tested. A DNA sequence in pTATA region was identified which confers ligand-independent activation of PSA promoter activity in C4-2 cells (see below). The size of the super PSA promoter is only 560bp which is significantly smaller than the 6Kb wild type PSA promoter, and it is also smaller than the 1071 bp PSE promoter. Yet, the super PSA promoter has 2-4 fold higher activity when compare to the wild type and ~5 fold higher activity when comparing with the PSE promoter. Moreover, super PSA promoter is highly inducible in LNCaP (52 fold) and C4-2 (16 fold). The high basal and androgen-induced super PSA promoter activity was observed in cells that expressed AR activity$_1$ suggesting that AR and a yet undefined co-factor(s) in C4-2 & LNCaP but not PC3 cells are responsible for upregulating PSA promoter activity (see below). However, the pTATA region alone could also be responsible for upregulating PSA promoter activity in C4-2 cells in an androgen- and AR-independent manner (see below). From this graph, it is clear that super PSA promoter has much higher activities in the AI C4-2 than AD LNCaP cells, this makes it a superior promoter to deliver and express genes in Al prostate cancer cells.

4 Example: Determination of the Complete Sequence of the Super-PSA Promoter

The complete sequence of the super-PSA promoter consists of the ARE enhancer core (AREc) that identified by Trapman (Stamey TA, Yang N, et al. *J Urol.* 141:1088-90) and a new pTATA element which is identified as described herein. The androgen inducibility resides within the ARE enhancer core region. In addition to the well-characterized androgen responsive element (ARE) III, other AREs have also been identified within the enhancer core recently (Lyss AP. (1987) *American J Med* 83:1120-27). The

androgen inducibility of AREc relies heavily on the AREIII and is specific to cells with endogenous PSA (Stamey TA, Yang N, et al. *J Urol.* 141:1088-90). It has been suggested that in the absence of androgen, AR could be signal through a ligand-independent pathway involving selected growth factors and intracellular signal transduction in androgen independent prostate cancer cells (Culig Z, Hobisch A, et al. (1994). *Cancer Res.* 54:5474-78); Nazarth LV, Wigel NL. (1996). *JBC* 271:19900-19907); Ruiter de PE, Twuwen R. et al. (1995) *Mol Cell endocrinol* 110:R1-6). AR could complexes with cofactor(s) and together could then bind to the AREc and transactivate PSA gene expression (Hsieh JT, Wu HC, et al. (1993) *Cancer Res* 52:2852-57). It is speculated that the regions surround the AREIII are the binding sites for these protein complexes. Therefore, any alterations of the regions may enhance or diminish the super-PSA promoter activity. The pTATA by itself is a strong element: when juxtaposed to the AREc it synegerizes with the enhancer core to give a superior promoter activity that surpasses the native PSA promoter. Through DNA footprinting experiment, 2 sites in pTATA were identified that are protected by protein factors in C4-2 and LNCAP nuclear extracts. Upon comparison with a sequence marker, the exact location of the protected regions were identified. One of the protected regions was named P2 which was extremely important for pTATA activity. The deletion of P2 eliminates most of the pTATA activity in prostate cancer cells. Data was obtained suggesting that specific transcription factor(s) binds to P2 which may interact with the general transcription machinery to enhance PSA gene expression in the absence of androgen. Furthermore, this interaction does not require neither androgen nor its receptor.

5 Example: Determination of the Binding of The Potential Protein Factors to the Radiolabeled P2 Element

In this example, the binding of the potential protein factors to the radiolabeled P2 element in an Electrophoretic~Mobility-Shift-Assay (EMSA) was determined. Due to their higher molecular weights, these protein-DNA complexes migrate slower than the free probes in the gel, producing distinct bands corresponds to their own molecular weight. LNCaP nuclear extract was used in lanes 1-5, C4-2 nuclear extract was used in lanes 6-10 and PC3 nuclear extract was used in lanes 11-13. Through consensus sites search, P2 appears to have high homology with SP-1 sites, so SP-1 sites were used to compare with the P2 site. In lanes 1,7 & 11, SP-1 site gives 3 distinct bands which correspond to the binding of SP1, SP-2 & SP-3 proteins, and these bands can be competed away specifically with non-labeled SP-I sites (lanes 2,6). With P2 site, there are several bands detected (lanes 3, 8 & 12). Bands marked with arrows are considered to be major

bands and none of them are similar to the bands produced by the SP-1 site. Moreover, non-radiolabeled SP-1 site was unable to compete away the protein-DNA complexes produce by P2 (lanes 4,9), while non-labeled P2 site can specifically compete away the protein-DNA complexes (lanes 5,10). Thus, it is reasonable to conclude that the P2 associating factors may not be SP-1 proteins. In addition, the two bands indicated in lanes 3 and 8 are more intense in C4-2 nuclear extract than LNCaP nuclear extract, suggesting there may be an upregulation of the factors in hormone independent C4-2 cells which in turn might enhance the PSA promoter activity in C4-2. With PC3 nuclear extract, P2 element also produces two major hands (lanes 12 & 13), but their sizes are clearly different from the bands of LNCaP (lane 3) and C4-2 (lane 8). It is possible that different: factors occupy the P2 site in PC3 cells but failed to upregulate PSA promoter activity. Therefore, the protein factor(s) associate with P2 site in the pTATA is specific to PSA-producing cell like C4-2 and LNCaP.

6 Example: Determination of the Basal Activity and the Androgen Inducibility of the Super PSA Promoter

In this example, the basal activity and the Androgen inducibility of the native and super PSA promoter were determined. The super PSA promoter consistently has a higher basal activity than the native PSA promoter in other prostate cell lines (Figure 5). NbE is a spontaneous immortalized normal rat prostate epithelial cell line which comprised a super PSA promoter activity ~10 fold higher than the native PSA promoter activity. The level of the super PSA promoter activity in NbE is similar to that of the AI C4-2 cells. PC3 is a human prostate cancer cell line. It does not have endogenous AR nor PSA, so the native PSA promoter activity has been conventionally lower. However, the super-PSA promoter is still capable of surpassing the native PSA promoter activity in PC3 by ~3 fold.

The androgen inducibility of the native and super PSA promoter in NbE & PC-3 cells is shown in Figure 6. When the androgen inducibility of the native and super PSA promoter in NbE & PC-3 cells from figure 6 is compared to figure 5, p61 but not AREc/pTATA promoter-mediated reporter activity in NbE and PC-3 cells can be further enhanced by an androgen agonist, R-1 881. Conversely, a somewhat reduced AREc/pTATA-mediated reporter activity exhibited in NbE and PC-3 cells subsequent to R-1881 exposure. The exact mechanism of these differential responses at the present time is unclear. It suffices to say, however, that super PSA promoter could drive the expression of

target genes in cells that are of prostate origin whether they produce PSA or not. Super PSA promoter, however, is much more active in prostate cells that contain AR (such as C4-2, LNCaP & NbE) than those that do not (such as PC-3).

WHAT IS CLAIMED IS:

1. An isolated polynucleotide comprising;

(a) the pTATA nucleotide sequence depicted in Figure 3,

(b) the pTATA nucleotide sequence depicted in Figure 3 juxtaposed to the AREc nucleotide sequence depicted in Figure 3, or

(c) a transcriptionally active fragment of the isolated polynucleotide of (a) or (b).

2. An isolated polynucleotide comprising the P2 nucleotide sequence depicted in Figure 3, or a transcriptionally active fragment thereof.

3. An isolated polynucleotide that hybridizes under highly stringent conditions to the complement of the polynucleotide of Claim 1.

4. An isolated polynucleotide that hybridizes under moderately stringent conditions to the complement of the polynucleotide of Claim 1.

5. An isolated polynucleotide that comprises the complement of the polynucleotide of Claim 1.

6. An isolated polynucleotide comprising the polynucleotide of Claims 1 or 2 operably associated with a heterologous coding sequence.

7. A vector comprising the polynucleotide of Claims 1, 2, 3 or 4.

8. An expression vector comprising the polynucleotide of Claims 1, 2, 3 or 4 operably associated with a heterologous coding sequence.

9. A genetically engineered host cell comprising the polynucleotide of Claims 1, 2, 3 or 4.

10. A genetically engineered host cell comprising the polynucleotide of Claims 1, 2, 3 or 4 operably associated with a heterologous coding sequence.

11. A transgenic, non-human animal comprising the polynucleotide of Claims 1, 2, 3 or 4.

12. The polynucleotide of claim 6, wherein the heterologous coding sequence is a reporter gene.

13. The polynucleotide of claim 12, wherein the reporter gene is luciferase.

14. A method for identifying a test compound capable of modulating prostate-specific gene expression comprising:

(a) measuring the level of expression of a reporter gene under the control of a super-PSA regulatory region, or a transcriptionally active fragment thereof, in the presence and absence of said test compound,

such that if the level obtained in the presence of the test compound differs from that obtained in its absence, then a compound which modulates prostate-specific gene expression is identified.

15. The method of claim 14 wherein the reporter gene is luciferase.

16. A pharmaceutical composition comprising the test compound identified by the method in claim 14.

17. A method for delivery of a therapeutic molecule comprising, introducing into prostate cancer cells of a subject a vector comprising a supre-PSA regulatory region sequence, or transcriptionally active fragment thereof, operatively linked to a heterologous nucleic acid which encodes said therapeutic molecule.

18. A method for inhibiting or treating prostate-related cancer or other proliferative disorder comprising introducing into a cell of a subject a vector comprising a super-PSA regulatory region sequence, or transcriptionally active fragment thereof, operatively linked to a heterologous nucleic acid whose gene product is capable of killing said cell.

19. A method for preventing or delaying a prostate-related disorder comprising introducing into a cell of a subject a vector comprising a super-PSA regulatory region sequence, or transcriptionally active fragment thereof, operatively linked to a heterologous nucleic acid which encodes a therapeutic molecule which is capable of preventing or delaying said disorder.

20. The method of Claim 19, wherein said disorder is prostate cancer.

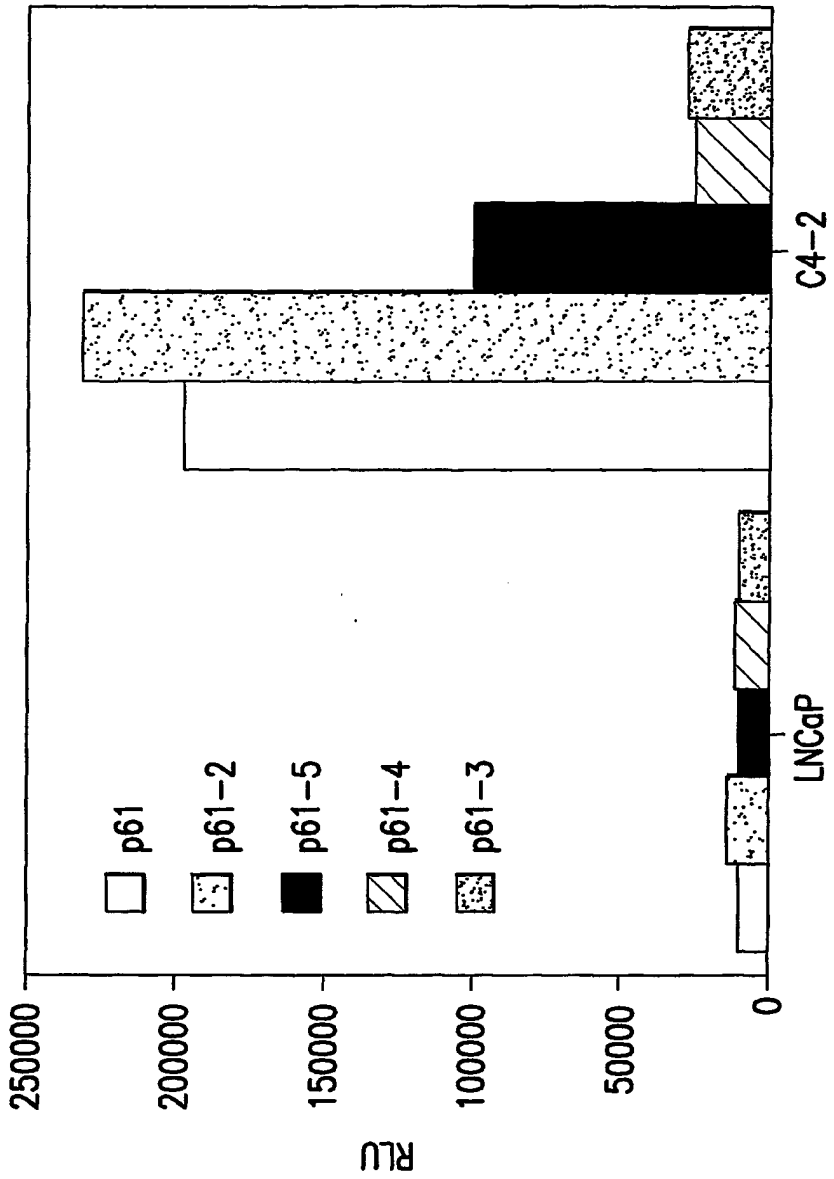

250000
200000
150000
100000
50000
0
RLU
p61
p61-2
p61-5
p61-4
p61-3
LNCaP
C4-2

FIG. 1A

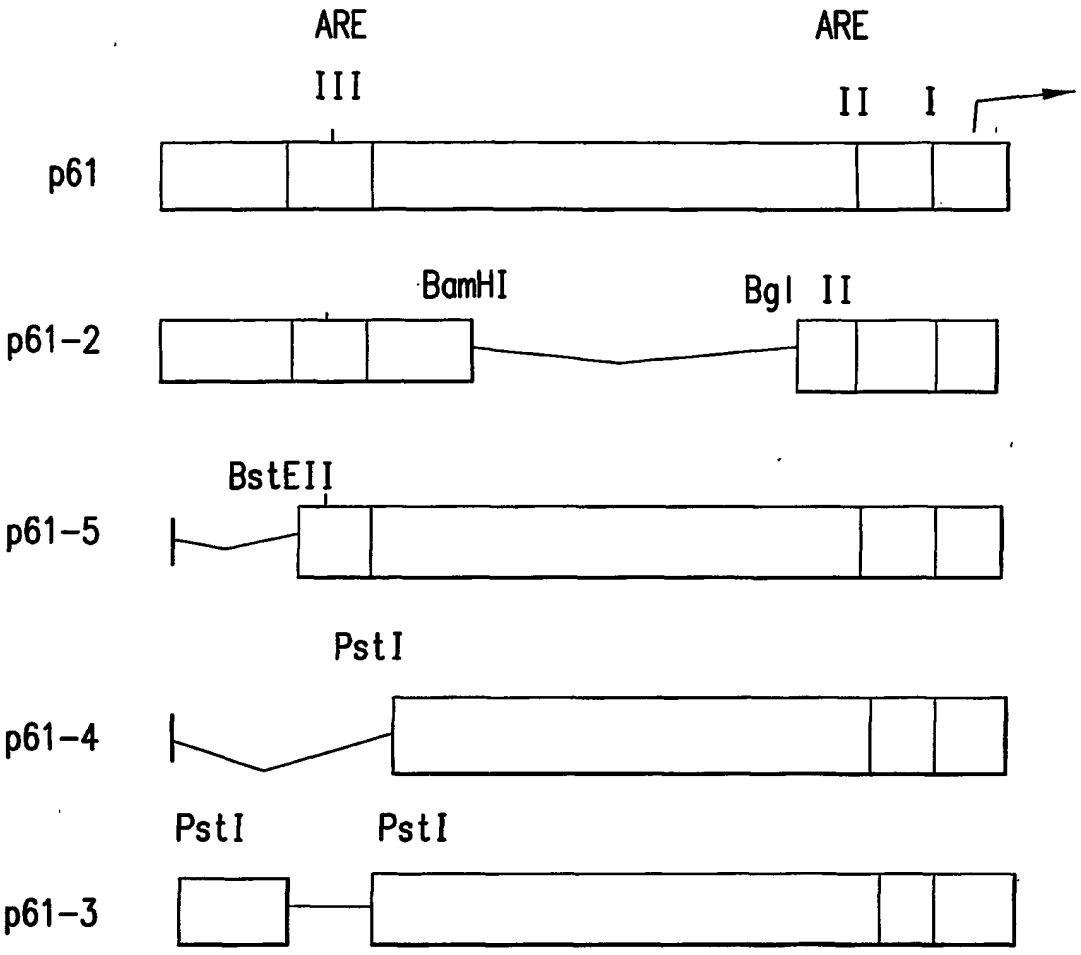

ARE
III
ARE
II
I
p61
BamHI
BgI II
p61-2
BstEII
p61-5
PstI
p61-4
PstI
PstI
p61-3

FIG. 1B

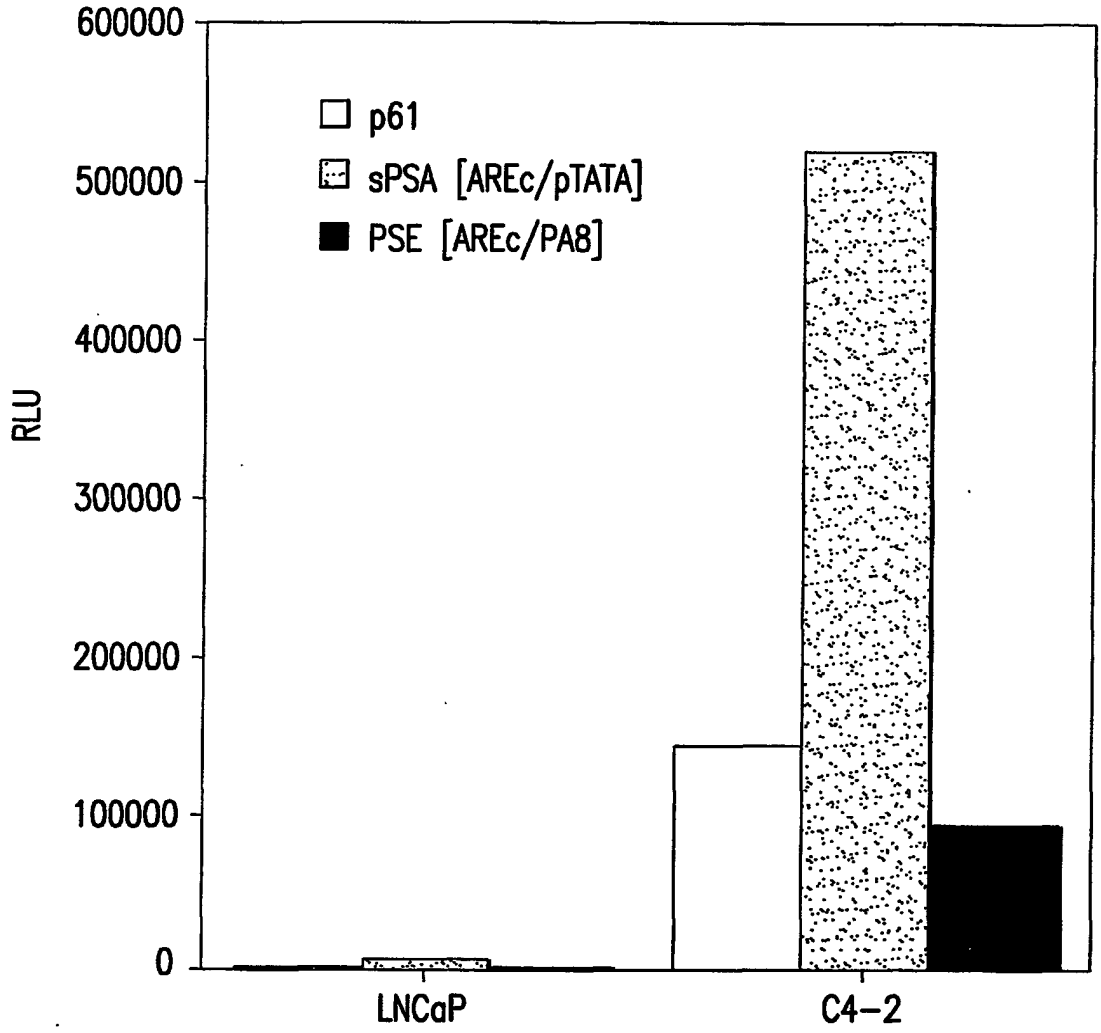

600000
500000
400000
300000
200000
100000
0
RLU
p61
sPSA [AREc/pTATA]
PSE [AREc/PA8]
LNCaP
C4-2

FIG.2

GGTGACCAGAGCAGTCTAGGTGGATGCTGTGCACACGGGGTTTGTGCCACTGGTGAGAAACCTGAA

AREc

GATTAGGAATCCTCAATCTTATACTGGACAACTGCAAGCCTGCTCAGCCTTTGTCTCGATGAAGAT

ARE III

ATTATCTTCATGATCTTGGATTGAAAACAGACCTACTCTGGAGGAACATATTGTATCGATTGTCTT

GACAGTAAACAAATCTGTTGTAAGAGACATTATCTTTATTATCTAGGACAGTAAGCAAGCCTGGAT

CTGAGAGAGATATCATCTTGCAAGGATGCCTGCTTTACAAACATCCTTGAAACAACAATCCAGAAA

AAAAAAGGTGTTGCTGTCTTTGCTCAGAAGACACACAGATACGTGACAGAACCATGGAGAATTGCC

TCCCAACGCTGTTCAGCCAGAGGCTTCCACCCTTGTCT

pTATA

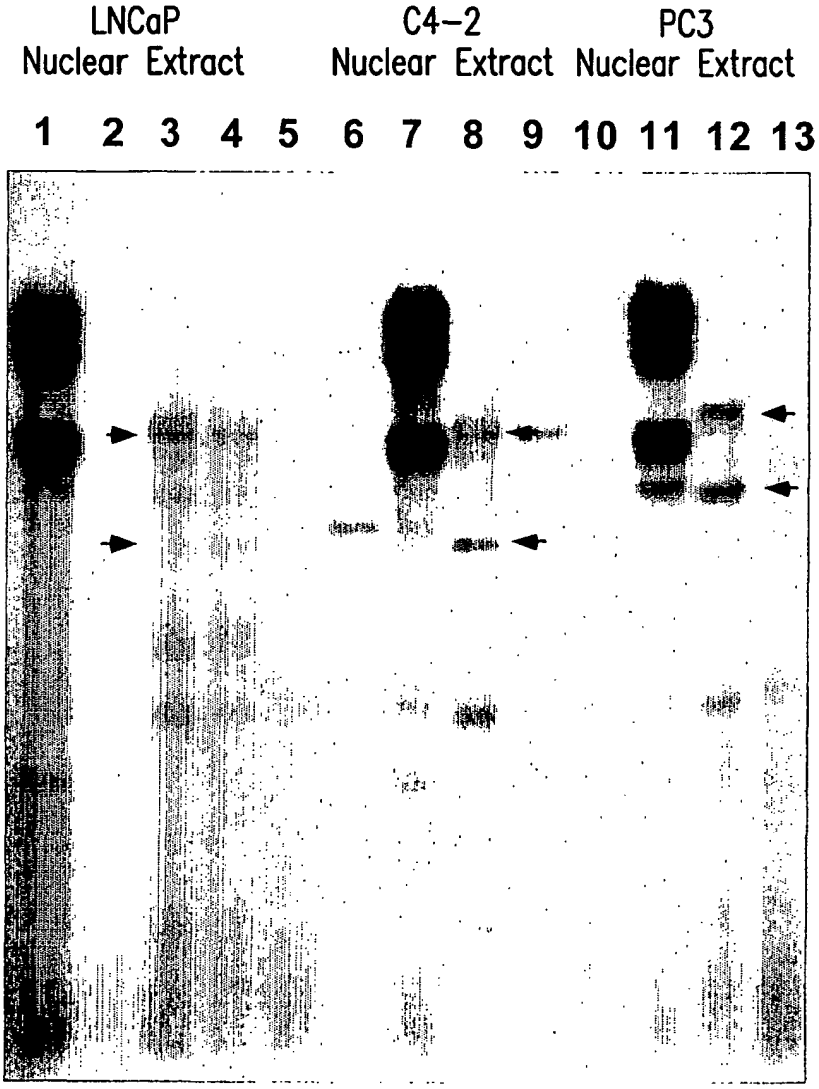
GCTAGCTCTCCCTCCCCTTCCACAGCTCTGGGTGTGGGAGGGGGTTGTCCAGCCTCCAGCAGCATC

P2

CCCAGGGCCTTGGTCAGCCTCTGGGTGCCAGCAGGGCAGGGGCGGAGTCCTGGGGAATGAAGGTTT

TATAGGGCTCCTGGGGGAGGCTCCCCAGCCCCAAGCTT

FIG.3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Nuclear Extract
C4-2
Nuclear Extract
PC3
Nuclear Extract
1 2 3 4 5 6 7 8 9 10 11 12 13

FIG.4

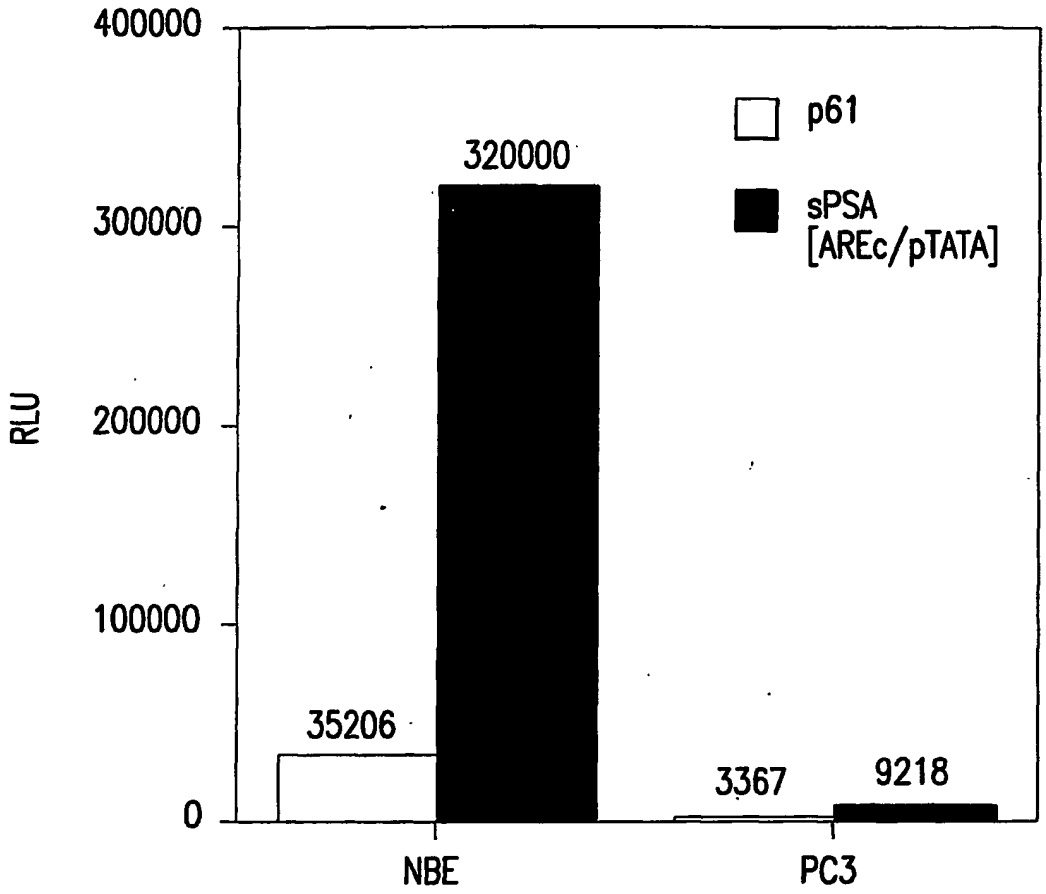
400000
300000
200000
100000
0
RLU
320000
35206
3367
9218
p61
sPSA
[AREc/pTATA]
NBE
PC3

FIG.5

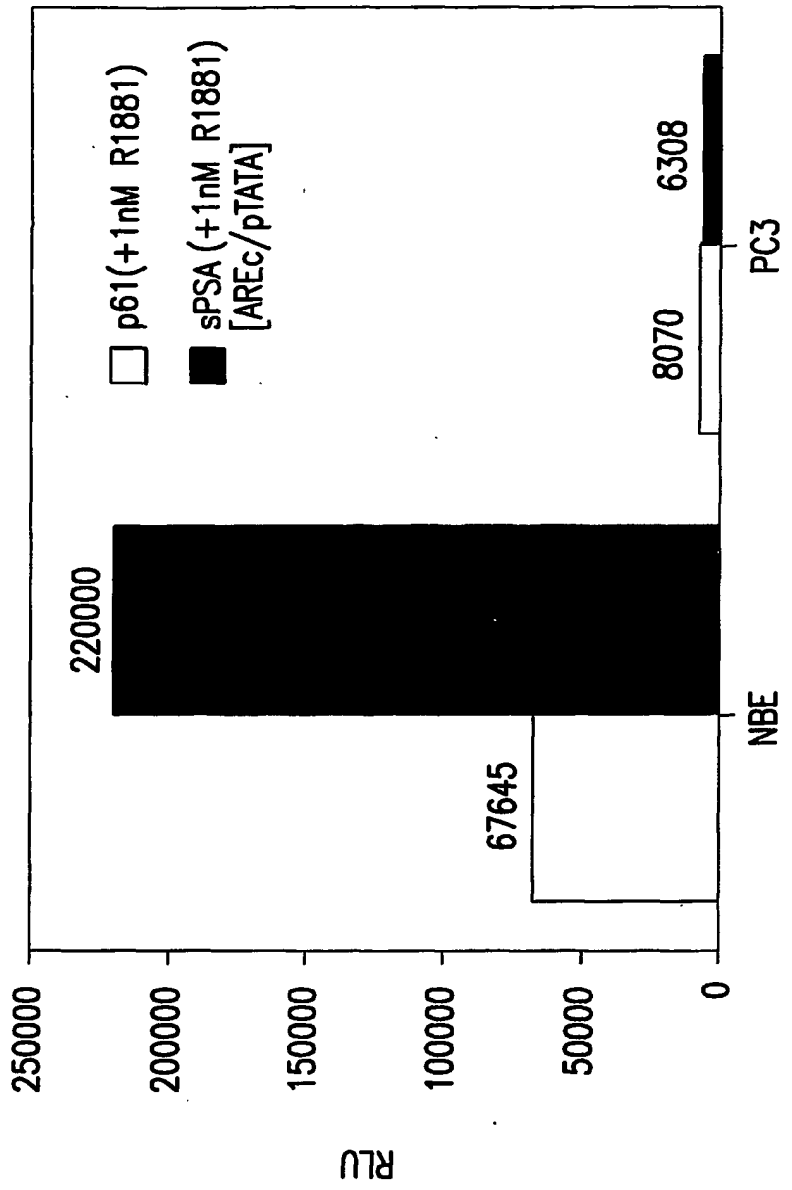
p61(+1nM R1881)
sPSA (+1nM R1881)
[AREc/pTATA]
220000
67645
8070
6308
NBE
PC3
250000
200000
150000
100000
50000
0
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FIG.6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/29581

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C07H 21/02, 21/04; C12Q 1/68; C12N 15/63, 15/00; A01N 43/04
US CL :536/23.1, 24.1; 435/6, 320.1, 455; 514/44
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.1, 24.1; 435/6, 320.1, 455; 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, STN, MEDLINE, BIOSIS, SCISEARCH, CAPLUS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CLEUTJENS et al. An Androgen Response Element in a Far Upstream Enhancer Region is Essential for High, Androgen-Regulated Activity of the Prostate-Specific Antigen Promoter. Molecular Endocrinology. February 1997, Vol. 11, No. 2, pages 148-161, especially page 148, 152, 159.	14-17
X	PANG et al. Identification of a Positive Regulatory Element Responsible for Tissue-Specific Expression of Prostate-Specific Antigen. Cancer Research. 01 February 1997, Vol. 57, pages 495-499, especially page 495.	14-17
X	WO 98/35031 A1 (UNIVERSITY OF ROCHESTER MEDICAL CENTER) 13 August 1998, entire document, especially page 2, 3, 11.	17-20

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/29581

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim N
X	WO 95/19434 A1 (CALYDON, INC.) 20 July 1995, entire document, especially abstract, page 34, 35.	17-20

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/29581

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1-13
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Please See Extra Sheet.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payme of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search repc covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/29581

BOX I. OBSERVATIONS WHERE CLAIMS WERE FOUND UNSEARCHABLE

2. Where no meaningful search could be carried out, specifically:

There are more than one nucleotide sequence depicted in Figure 3 and no SEQ ID No. is provided. It is unclear what nucleotide sequence is intended in claim 1. It is also unclear what is the difference between "the pTATA nucleotide sequence depicted in Figure 3" and "the pTATA nucleotide sequence depicted in Figure 3 juxtaposed to the AREc nucleotide sequence depicted in Figure 3". No sequence search could be performed.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

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(54) Title: TRP8, TRP9 AND TRP10, NOVEL MARKERS FOR CANCER

(57) Abstract: The present invention relates to gene expression in normal cells and cells of malignant tumors and particularly to novel markers associated with cancer, Trp8, Trp9 and Trp10, and the genes encoding Trp8, Trp9 and Trp10. Also provided are vectors, host cells, antibodies, and recombinant methods for producing these human proteins. The invention further relates to diagnostic and therapeutic methods useful for diagnosing and treating a tumor.

Trp8, Trp9 and Trp10, novel markers for cancer

FIELD OF THE INVENTION

The present invention relates to gene expression in normal cells and cells of malignant tumors and particularly to novel markers associated with cancer, Trp8, Trp9 and Trp10, and the genes encoding Trp8, Trp9 and Trp10

BACKROUND OF THE TECHNOLOGY

Prostate cancer is one of the most common diseases of older men world wide. Diagnosis and monitoring of prostate cancer is difficult because of the heterogeneity of the disease. For diagnosis different grades of malignancy can be distinguished according to the Gleason-Score Diagnosis. For this diagnosis a prostate tissue sample is taken from the patient by biopsy and the morphology of the tissue is investigated. However, this approach only yields subjective results depending on the experience of the pathologist. For confirmation of these results and for obtaining an early diagnosis an additional diagnostic method can be applied which is based on the detection of a prostate specific antigen (PSA). PSA is assayed in serum samples, blood samples etc. using an anti-PSA-antibody. However, since in principle PSA is also expressed in normal prostate tissue there is a requirement for the definition of a threshold value (about 4 ng/ml PSA) in order to be able to distinguish between normal and malign prostate tissue. Unfortunately, this diagnostic method is quite insensitive and often yields false-positive results. Moreover, by using this diagnostic method any conclusions as regards the grade of malignancy, the progression of the tumor and its potential for metastasizing cannot be drawn. Thus, the use of molecular markers would be helpful to distinguish benign from malign tissue and for grading and staging prostate carcinoma, particularly for patients with metastasizing prostate cancer having a very bad prognosis.

The above discussed limitations and failings of the prior art to provide meaningful specific markers which correlate with the presence of prostate tumors, in particular metastasizing tumors, has created a need for markers which can be used diagnostically, prognostically and therapeutically over the course of this disease. The present invention fulfils such a need by the provision of Tpr8, Trp9 and Trp10 and the genes encoding Trp8, Trp9 and Trp10: The genes encoding Trp8 and Trp10 are expressed in prostate carcinoma and prostatic metastasis, but

not in normal prostate, benign hyperplasia (BHP) and intraepithelial prostatic neoplasia (PIN). Furthermore, expression of Trp10 transcripts is detectable in carcinoma but not in healthy tissue of the lung, the prostate, the placenta and in melanoma.

SUMMARY OF THE INVENTION

The present invention is based on the isolation of genes encoding novel markers associated witha cancer, Trp8, Trp9 and Trp10. The new calcium channel proteins Trp8, Trp9 and Trp10 are members of the trp (transient receptor potential) - family, isolated from human placenta (Trp8a and Trp8b) and humane prostate (Trp9, Trp10a and Trp10b). Trp proteins belong to a steadily growing family of Ca^{2+} selective and non selective ion channels. In the recent years seven Trp proteins (trp1 - trp7) have been identified and suggested to be involved in cation entry, receptor operated calcium entry and pheromone sensory signaling. Structurally related to the trp proteins are the vanilloid receptor (VR1) and the vanilloid like receptor (VRL-1) both involved in nociception triggered by heat. Furthermore, two calcium permeable channels were identified in rat small intestine (CaT1) and rabbit kidney (ECaC). These distantly related channels are suggested to be involved in the uptake of calcium ions from the lumen of the small intestine (CaT1) or in the reuptake of calcium ions in the distal tubule of the kidney (ECaC). Common features or the Trp and related channels are a proposed structure comprising six transmembrane domains including several conserved amino acid motifs. In the present invention the cloning and expression of a CaT1 like calcium channel (Trp8) from human placenta as well as Trp9 and Trp10 (two variants, Trp10a and Trp10b) is described. Two polymorphic variants of the Trp8 cDNA were isolated from placenta (Trp8a and Trp8b). Transient expression of the Trp8b cDNA in HEK (human embryonic kidney) cells results in cytosolic calcium overload implicating that the Trp8 channel is constitutive open in the expression system. Trp8 induces highly calcium selective inward currents in HEK cells. The C -terminus of the Trp8 protein binds calmodulin in a calcium dependent manner. The Trp9 channel is expressed in trophoblasts and syncytiotrophoblasts of placenta and in pancreatic acinar cells. Furthermore, the Trp8 channel is expressed in prostatic carcinoma and prostatic metastases, but not in normal tissue of the prostate. No expression of Trp8 transcripts is detectable in benign prostatic hyperplasia (BPH) or prostatic intraepithelial neoplasia (PIN). Therefore, the Trp8 channel is exclusively expressed in malign prostatic tissues and serves as molecular marker for prostate cancer. From the experimental results it is also apparent that the

modulation of Trp8 and/or Trp10, e.g. the inhibition of expression or activity , is of therapeutic interest, e.g. for the prevention of tumor progression.

The present invention, thus, provides a Trp8, Trp9 and Trp10 protein, respectively, as well as nucleic acid molecule encoding the protein and, moreover, an antisense RNA, a ribozyme and an inhibitor, which allow to inhibit the expression or the activity of Trp8, Trp9 and/or Trp10.

In one embodiment, the present invention provides a diagnostic method for detecting a prostate cancer or endometrial cancer (cancer of the uterus) associated with Trp8 or Trp10 in a tissue of a subject, comprising contacting a sample containing Trp8 and/or Trp10 encoding mRNA with a reagent which detects Trp8 and/or Trp10 or the corresponding mRNA.

In a further embodiment, the present invention provides a diagnostic method for detecting a melanoma, chorion carcinoma, cancer of the lung and of the prostate in a tissue of a subject, comprising contacting a sample with a reagent which detects Trp10a and/or Trp10b antisense transcripts or Trp10a and/or Trp10b related antisense transcripts.

In another embodiment, the present invention provides a method of treating a prostate tumor, carcinoma of the lung, carcinoma of the placenta (chorion carcinoma) or melanoma associated with Trp8 and/or Trp10, comprising administering to a subject with such an disorder a therapeutically effect amount of a reagent which modulates, e.g. inhibits, expression of Trp8 and/or Trp10 or the activity of the protein, e.g. the above described compounds.

Finally, the present invention provides a method of gene therapy comprising introducing into cells of a subject an expression vector comprising a nucleotide sequence encoding the above mentioned antisense RNA or ribozyme, in operable linkage with a promoter.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: A, phylogenetic relationship of trp and related proteins. B, hydropathy plot of the Trp8 protein sequence according to Kyte and Doolittle. C, alignment of Trp8a/b to the epithelial calcium channels ECaC (from rabbit) and Vr1 (from rat). Putative transmembrane domains are underlined.

Figure 2: A, polymorphism of the Trp8 gene. The polymorphic variants Trp8a and Trp8b differ in five base pairs resulting in three amino acid exchanges in the derived protein sequences. Specific primers were derived from the Trp8 gene as indicated by arrows. B, the Trp8a and Trp8b genes are distinguishable by a single restriction site. Genomic fragments of the Trp8 gene can be amplified using specific primers (shown in A). The genomic fragment of the Trp8b gene contains an additional site of the restriction enzyme BSP1286I (B). C, the Trp8 gene is located on chromosome 7. D, genotyping of eleven human subjects. A 458 bp genomic fragment of the Trp8 gene was amplified using specific primers (shown in A) and restricted with BSP1286I. The resulting fragments were analyzed by PAGE electrophoresis.

Figure 3: The Trp8b protein is a calcium selective ion channel. A, representative trace of a pdiTrp8b transfected HEK 293 cell. Trp8b mediated currents are activated by voltage ramps (-100 mV - +100 mV) of 100 msec at -40 mV or +70 mV holding potential. 1, Trp8b currents in the presence at 2mm $[Ca^{2+}]_o$;2, effect of solution switch alone 3, switch to nominal zero calcium solution. B, Trp8b currents in the presence of zero divalent cations. C, current voltage relationship of the currents shown in A. Inset, leak subtracted current. D, current voltage relationship of the current shown in B. E, statistics of representative experiments. Black: Trp8 transfected cells, gray: control cells. Columns from left to right: Trp8 currents at - 40 mV (n =12) and + 70 mV holding potential (n = 12). Trp8 currents in standard bath solution including 120 mM NMDG without sodium (n = 7) and with nominal zero calcium ions (n = 8) or in the presence of 1mM EGTA with zero divalent cations (n = 6). F, representative changes in $[Ca^{2+}]_i$ in Trp8b transfected HEK cells (gray) and controls (black) in the presence or absence of 1mM $[Ca^{2+}]_o$. Inset, relative increase of cytosolic calcium concentration of Trp8b transfected HEK cells, before and after readdition of 1 mM $[Ca^{2+}]_o$ in comparison to control cells.

Figure 4: The C-terminal region of the Trp8 protein binds calmodulin. A, N- and C-terminal fragments of the Trp8 protein used for calmodulin binding studies. B, the Trp8 protein and a truncated Trp8 protein which was in vitro translated after MunI cut of the cDNA, which lacks the C-terminal 32 amino acid residues, were in vitro translated in the presence of ^{35}S-methionine and incubated with calmodulin coupled agarose beads in the presence of 1 mM Ca^{2+} or 2 mM EGTA. C, calmodulin binding to N- and C-terminal fragments of the Trp8protein in the presence of Ca^{2+} (1 mM) or EGTA (2 mM)

Figure 5: Expression pattern of the Trp8 cDNA. A, Northern blots (left panels, Clontech, Palo Alto) were hybridized using a 348 bp NcoI/BamHT fragment of the Trp9 cDNA. The probe hybridizes to mRNA species isolated from the commercial blot, but not to mRNA species isolated from benign prostate hyperplasia (right panel, mRNA isolated from 20 human subjects with benign prostate hyperplasia). B,C, in situ hybridization with biotinylated Trp8 specific oligonucleotides on slides of human tissues. Left column antisense probes, right column sense probes. D, antinsense probes.

Figure 6: Differential expression of Trp8 cDNA in human prostate. A - F, in situ hybridization with prostatic tissues. A, normal prostate, B, primary carcinoma, C, benign hyperplasia, D, rezidive carcinoma, E, prostatic intraepithelial neoplasia, F, lymphnode metastasis of the prostata.

Figure 7: Trp8a cDNA sequence and derived amino acid sequence

Figure 8: A, Trp8b cDNA sequence and derived amino acid sequence
B, cDNA sequence of splice variant 1 (12B1)
C, cDNA sequence of splice variant 2 (17-3)
D, cDNA sequence of splice variant 3 (23A3)
E, cDNA sequence of splice variant 4 (23C3)

Figure 9: A, Trp9 cDNA sequence and derived amino acid sequence B, cDNA sequence of splice variant 15 and derived amino acid sequence.

Figure 10: A, cDNA sequence of Trp10a and derived amino acid sequence, B, cDNA fragment of Trp10a and derived amino acid sequence.

Figure 11: cDNA sequence of Trp10b and derived amino acid sequence.

Figure 12: Expression of Trp8 mRNA in human endometrial cancer or cancer of the uterus. A - D, in situ hybridization with slides of endometrial cancer hybridized with Trp8 antisense (left column) or sense probes as controls (right column). E - F, Trp8 antisense probes hybridized to slides of normal endometrium. It can be clearly seen no hybridization occurs with normal endometrial tissue.

Figure 13: Expression of human Trp9 and Trp10 genes
Northern blots were hybridized using Trp9 (upper panel) or Trp10 (lower panel) specific probes. Expression of the Trp9 cDNA is detectable in many tissues including human prostate and colon as well as in benign prostatic hyperplasia. Expression of Trp10 cDNA is detectable in human prostate of a commercial northern blot (Clontech, right side). This Northern blot contains prostatic tissue collected from 15 human subjects in the range of 14 - 60 years of age. No expression of Trp10 cDNA was detectable in benign prostatic hyperplasia (left side).

Figure 14: Expression of Trp10 transcripts and Trp10-antisense transcripts in human prostate cancer and metastasis of a melanoma. In situ hybridizations of slides hybridized with Trp10-antisense (A-E, K-N) and Trp10 related sense probes (F-J, P-R). It can clearly be seen that both probes detect the same cancer cells indicating that these cancer cells express Trp10 transcripts as well as Trp10-antisense transcripts. S, no Trp10 expression is detectable in benign hyperplasia of the prostate (BPH). O and T, show expression of Trp10 transcripts (O) and Trp10-antisense transcripts (T) in a metastasis of a melanoma in human lung. Melanoma cancer cells express both Trp10 transcripts and Trp10-antisense transcripts.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to an isolated nucleic acid molecule encoding the human prostate carcinoma associated protein Trp8a, Trp8b, Trp9, Trp10a or Trp10b or a protein exhibiting biological properties of Trp8a, Trp8b, Trp9, Trp10a or Trp10b and being selected from the group consisting of

(a) a nucleic acid molecule encoding a protein that comprises the amino acid sequence depicted in Figure 7, 8A, 9,10 or 11;

(b) a nucleic acid molecule comprising the nucleotide sequence depicted in Figure 7, 8A, 9,10, or 11;

(c) a nucleic acid molecule included in DSMZ Deposit no. DSM 13579 (deposit date: 28 June 2000), DSM 13580 (deposit date: 28 June 2000), DSM 13584 (deposit date: 5 July 2000), DSM 13581 (deposit date: 28 June 2000) or DSM ....(deposit date:....);

(d) a nucleic acid molecule with hybridizes to a nucleic acid molecule specified in (a) to (c)

(e) a nucleic acid molecule the nucleic acid sequence of which deviates from the nucleic sequences specified in (a) to (d) due to the degeneration of the genetic code; and

(f) a nucleic acid molecule, which represents a fragment, derivative or allelic variation of a nucleic acid sequence specified in (a) to (e).

As used herein, a protein exhibiting biological properties of Trp8a, Trp8b, Trp9,Trp10a or Trp10b is understood to be a protein having at least one of the activities as illustrated in the Examples, below.

As used herein, the term „isolated nucleic acid molecule„ includes nucleic acid molecules substantially free of other nucleic acids, proteins, lipids, carbohydrates or other materials with which it is naturally associated.

In a first embodiment, the invention provides an isolated nucleic acid molecule encoding the human prostate carcinoma associated protein Trp8a, Trp8b, Trp9,Trp10a or Trp10b comprising the amino acid sequence depicted in Figure 7, 8A, 9,10 or 11. The present invention also provides a nucleic acid molecule comprising the nucleotide sequence depicted in Figure 7, 8A, 9,10 or 11.

The present invention provides not only the generated nucleotide sequence identified in Figure 7, 8A, 9,10 or 11, respectively and the predicted translated amino acid sequence, respectively, but also plasmid DNA containing a Trp8a cDNA deposited with the DSMZ, under DSM 13579, a Trp8b cDNA deposited with the DSMZ, under DSM 13580, a Trp9 cDNA deposited with the DSMZ, under DSM 13584, a Trp10a cDNA deposited with the DSMZ, under DSM 13581, and a Trp10b cDNA deposited with the DSMZ, under DSM...., respectively. The nucleotide sequence of each deposited Trp-clone can readily be determined by sequencing the deposited clone in accordance with known methods. The predicted amino acid sequence can then be verified from such deposits. Moreover, the amino acid sequence of the protein encoded by each deposited clone can also be directly determined by peptide sequencing or by expressing the protein in a suitable host cell containing the deposited Trp-encoding DNA, collecting the protein, and determining its sequence.

The nucleic acid molecules of the invention can be both DNA and RNA molecules. Suitable DNA molecules are, for example, genomic or cDNA molecules. It is understood that all

nucleic acid molecules encoding all or a portion of Trp8a, Trp8b, Trp9,Trp10a or Trp10b are also included, as long as they encode a polypeptide with biological activity. The nucleic acid molecules of the invention an be isolated from natural sources or can be synthesized according to know methods.

The present invention also provides nucleic acid molecules which hybridize to the above nucleic acid molecules. As used herein, the term „hybridize„ has the meaning of hybridization under conventional hybridization conditions, preferably under stringent conditions as described, for example, in Sambrook et al., Molecular Cloning, A Laboratory Manual 2nd edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. Also contemplated are nucleic acid molecules that hybridize to the Trp nucleic acid molecules at lower stringency hybridization conditions. Changes in the stringency of hybridization and signal detection are primarily accomplished through the manipulation of formamide concentration (lower percentages of formamide result in lowered stringency), salt conditions, or temperature. For example, lower stringency conditions include an overnight incubation at 37°Cin a solution comprising 6X SSPE (20X SSPE = 3M NaCl; 9.2M NaH_2PO_4; 0.02M EDTA, pH7.4), 0.5% SDS, 30% formamide, 100 µg/ml salmon sperm blocking DNA, following by washes at 50°C with 1 X SSPE, 0.1% SDS. In addition, to achieve even lower stringency, washes performed following stringent hybridization can be done at higher salt concentrations (e.g. 5X SSC). Variations in the above conditions may be accomplished through the inclusion and/or substitution of alternate blocking reagents used to suppress background in hybridization experiments. The inclusion of specific blocking reagents may require modification of the hybridization conditions described above, due to problems with compatibility.

Nucleic acid molecules that hybridize to the molecules of the invention can be isolated, e.g., from genomic or cDNA libraries that were produced from human cell lines or tissues. In order to identify and isolate such nucleic acid molecules the molecules of the invention or parts of these molecules or the reverse complements of these molecules can be used, for example by means of hybridization according to conventional methods (see, e.g., Sambrook et al., supra). As a hybridization probe nucleic acid molecules can be used, for example, that have exactly or basically the nucleotide sequence depicted in Figure 7, 8A, 9,10 or 11, respectively, or parts of these sequences. The fragments used as hybridization probe can be synthetic

fragments that were produced by means of conventional synthetic methods and the sequence of which basically corresponds to the sequence of a nucleic acid molecule of the invention.

The nucleic acid molecules of the present invention also include molecules with sequences that are degenerate as a result of the genetic code.

In a further embodiment, the present invention provides nucleic acid molecules which comprise fragments, derivatives and allelic variants of the nucleic acid molecules described above encoding a protein of the invention. „Fragments„ are understood to be parts of the nucleic acid molecules that are long enough to encode one of the described proteins. These fragments comprise nucleic acid molecules specifically hybridizing to transcripts of the nucleic acid molecules of the invention. These nucleic acid molecules can be used, for example, as probes or primers in the diagnostic assay and/or kit described below and, preferably, are oligonucleotides having a length of at least 10, in particular of at least 15 and particularly preferred of at least 50 nucleotides. The nucleic acid molecules and oligonucleotides of the invention can also be used, for example, as primers for a PCR reaction. Examples of particular useful probes (primers) are shown in Tables 1 and 2.

Table 1

Trp8 probes used for in situ hybridization:

Probes (antisense)

1.) 5' TCCGCTGCCGGTTGAGATCTTGCC 3'

2.) 5' CTTGCTCCATAGGCAGAGAATTAG 3'

3.) 5' ATCCTCAGAGCCCCGGGTGTGGAA3'

Controls (sense)

1.) 5' GGCAAGATCTCAACCGGCAGCGGA 3'

2.) 5' CTAATTCTCTGCCTATGGAGCAAG 3'

3.) 5' TTCCACACCCGGGGCTCTGAGGAT 3'

Tabelle 2

Trp10 probes used for the in situ hybridizations shown in Figure 14:

Probes (antisense)

1.) 5' GCTTCCACCCCAAGCTTCACAGGAATAGA 3' (Figure 14 A, 14B)

2.) 5' GGCGATGAAATGCTGGTCTGTGGC 3' (Figure 14C, 14D, 14N, 14S, 14O)

3.) 5' ATCTTCCAGTTCTTGGTGTCTCGG 3' (Figure 14E, 14K)

4.) 5' GCTGCAGTACTCCTGCACCAGGAA 3' (Figure 14L, 14M)

Probes (sense)

1.) 5' TCTATTCCTGTGAAGCTTGGGGTGGAAGC 3' (Figure 14F, 14G)

2.) 5' GCCACAGACCAGCATTTCATCGCC 3' (Figure 14H, 14I, 14T)

3.) 5' CCGAGACACCAAGAACTGGAAGAT 3' (Figure 14J, 14P)

4.) 5' TTCCTGGTGCAGGAGTACTGCAGC 3' (Figure 14Q, 14R)

The term „derivative„ in this context means that the sequences of these molecules differ from the sequences of the nucleic acid molecules described above at one or several positions but have a high level of homology to these sequences. Homology hereby means a sequence identity of at least 40%, in particular an identity of at least 60%, preferably of more than 80% and particularly preferred of more than 90%. These proteins encoded by the nucleic acid molecules have a sequence identity to the amino acid sequence depicted in Figure 7, 8A, 9, 10 and 11, respectively, of at least 80%, preferably of 85% and particularly preferred of more than 90%, 97% and 99%. The deviations to the above-described nucleic acid molecules may have been produced by deletion, substitution, insertion or recombination. The definition of the derivatives also includes splice variants, e.g. the splice variants shown in Figures 8B to 8E and 9B.

The nucleic acid molecules that are homologous to the above-described molecules and that represent derivatives of these molecules usually are variations of these molecules that represent modifications having the same biological function. They can be naturally occurring variations, for example sequences from other organisms, or mutations that can either occur naturally or that have been introduced by specific mutagenesis. Furthermore the variations can be synthetically produced sequences. The allelic variants can be either naturally occurring variants or synthetically produced variants or variants produced by recombinant DNA processes.

Generally, by means of conventional molecular biological processes it is possible (see, e.g., Sambrook et al., supra) to introduce different mutations into the nucleic acid molecules of the invention. As a result Trp proteins or Trp related proteins with possibly modified biological properties are synthesized. One possibility is the production of deletion mutants in which nucleic acid molecules are produced by continuous deletions from the 5'- or 3'-terminal of the coding DNA sequence and that lead to the synthesis of proteins that are shortened accordingly. Another possibility is the introduction of single-point mutation at positions where a modification of the amino aid sequence influences, e.g., the ion channel properties or the regulations of the trp-ion channel. By this method muteins can be produced, for example, that possess a modified ion conducting pore, a modified K_m-value or that are no longer subject to the regulation mechanisms that normally exist in the cell, e.g. with regard to allosteric regulation or covalent modification. Such muteins might also be valuable as therapeutically useful antagonists of Trp8a, Trp8b, Trp9,Trp10a or Trp10b, respectively.

For the manipulation in prokaryotic cells by means of genetic engineering the nucleic acid molecules of the invention or parts of these molecules can be introduced into plasmids allowing a mutagenesis or a modification of a sequence by recombination of DNA sequences. By means of conventional methods (cf. Sambrook et al., supra) bases can be exchanged and natural or synthetic sequences can be added. In order to link the DNA fragments with each other adapters or linkers can be added to the fragments. Furthermore, manipulations can be performed that provide suitable cleavage sites or that remove superfluous DNA or cleavage sites. If insertions, deletions or substitutions are possible, in vitro mutagenesis, primer repair, restriction or ligation can be performed. As analysis method usually sequence analysis, restriction analysis and other biochemical or molecular biological methods are used.

The proteins encoded by the various variants of the nucleic acid molecules of the invention show certain common characteristics, such as ion channel activity, molecular weight, immunological reactivity or conformation or physical properties like the electrophoretical mobilty, chromatographic behavior, sedimentation coefficients, solubility, spectroscopic properties, stability; pH optimum, temperature optimum.

The invention furthermore relates to vectors containing the nucleic acid molecules of the invention. Preferably, they are plasmids, cosmids, viruses, bacteriophages and other vectors

usually used in the field of genetic engineering. Vectors suitable for use in the present invention include, but are not limited to the T7-based expression vector for expression in mammalian cells and baculovirus-derived vectors for expression in insect cells. Preferably, the nucleic acid molecule of the invention is operatively linked to the regulatory elements in the recombinant vector of the invention that guarantee the transcription and synthesis of an RNA in prokryotic and/or eukaryotic cells that can be translated. The nucleotide sequence to be transcribed can be operably linked to a promoter like a T7, metallothionein I or polyhedrin promoter.

In a further embodiment, the present invention relates to recombinant host cells transiently or stable containing the nucleic acid molecules or vectors or the invention. A host cell is understood to be an organism that is capable to take up *in vitro* recombinant DNA and, if the case may be, to synthesize the proteins encoded by the nucleic acid molecules of the invention. Preferably, these cells are prokaryotic or eukaryotic cells, for example mammalian cells, bacterial cells, insect cells or yeast cells. The host cells of the invention are preferably characterized by the fact that the introduced nucleic acid molecule of the invention either is heterologous with regard to the transformed cell, i.e. that it does not naturally occur in these cells, or is localized at a place in the genome different from that of the corresponding naturally occurring sequence.

A further embodiment of the invention relates to isolated proteins exhibiting biological properties of the human prostate carcinoma associated protein Trp8a, Trp8b, Trp9,Trp10a or Trp10b and being encoded by the nucleic acid molecules of the invention, as well as to methods for their production, whereby, e.g., a host cell of the invention is cultivated under conditions allowing the synthesis of the protein and the protein is subsequently isolated from the cultivated cells and/or the culture medium. Isolation and purification of the recombinantly produced proteins may be carried out by conventional means including preparative chromatography and affinity and immunological separations involving affinity with an anti-Trp8a-, anti-Trp8b-, anti-Trp9-,anti-Trp10a- or anti-Trp10b-antibody, respectively.

As used herein, the term „isolated protein„ includes proteins substantially free of other proteins, nucleic acids, lipids, carbohydrates or other materials with which it is naturally associated. Such proteins however not only comprise recombinantly produced proteins but include isolated naturally occurring proteins, synthetically produced proteins, or proteins

produced by a combination of these methods. Means for preparing such proteins are well understood in the art. The Trp proteins are preferably in a substantially purified form. A recombinantly produced version of a human prostate carcinoma associated protein Trp8a, Trp8b, Trp9,Trp10a or Trp10b protein, including the secreted protein, can be substantially purified by the one-step method described in Smith and Johnson, Gene 67; 31-40 (1988).

In a further preferred embodiment, the present invention relates to an antisense RNA sequence characterised that it is complementary to an mRNA transcribed from a nucleic acid molecule of the present invention or a part thereof and can selectively bind to said mRNA, said sequence being capable of inhibiting the synthesis of the protein encoded by said nucleic acid molecules, and a ribozyme characterised in that it is complementary to an mRNA transcribed from a nucleic acid molecule of the present invention or a part thereof and can selectively bind to and cleave said mRNA, thus inhibiting the synthesis of the proteins encoded by said nucleic acid molecules. Riboyzmes which are composed of a single RNA chain are RNA enzymes, i.e. catalytic RNAs, which can intermolecularly cleave a target RNA, for example the mRNA transcribed from one of the Trp genes. It is now possible to construct ribozymes which are able to cleave the target RNA at a specific site by following the strategies described in the literature. (see, e.g., Tanner et al., in: Antisense Research and Applications, CRC Press Inc. (1993), 415-426). The two main requirements for such ribozymes are the catalytic domain and regions which are complementary to the target RNA and which allow them to bind to its substrate, which is a prerequisite for cleavage. Said complementary sequences, i.e., the antisense RNA or ribozyme, are useful for repression of Trp8a-, Trp8b, Trp9-,Trp10a- and Trp10b-expression, respectively, i.e. in the case of the treatment of a prostate cancer or endometrial cancer (carcinoma of the uterus). Preferably, the antisense RNA and ribozyme of the invention are complementary to the coding region. The person skilled in the art provided with the sequences of the nucleic acid molecules of the present invention will be in a position to produce and utilise the above described antisense RNAs or ribozymes. The region of the antisense RNA and ribozyme, respectively, which shows complementarity to the mRNA transcribed from the nucleic acid molecules of the present invention preferably has a length of at least 10, in particular of at least 15 and particularly preferred of at least 50 nucleotides.

In still a further embodiment, the present invention relates to inhibitors of Trp8a, Trp8b, Trp9, Trp10a and Trp10b, respectively, which fulfill a similar purpose as the antisense RNAs or

ribozymes mentioned above, i.e. reduction or elimination of biologically active Trp8a, Trp8b, Trp9, Trp10a or Trp10b molecules. Such inhibitors can be, for instance, structural analogues of the corresponding protein that act as antagonists. In addition, such inhibitors comprise molecules identified by the use of the recombinantly produced proteins, e.g. the recombinantly produces protein can be used to screen for and identify inhibitors, for example, by exploiting the capability of potential inhibitors to bind to the protein under appropriate conditions. The inhibitors can, for example, be identified by preparing a test mixture wherein the inhibitor candidate is incubated with Trp8a, Trp8b, Trp9, Trp10a or Trp10b, respectively, under appropriate conditions that allow Trp8a, Trp8b, Trp9, Trp10a or Trp10b to be in a native conformation. Such an in vitro test system can be established according to methods well known in the art. Inhibitors can be identified, for example, by first screening for either synthetic or naturally occurring molecules that bind to the recombinantly produced Trp protein and then, in a second step, by testing those selected molecules in cellular assays for inhibition of the Trp protein, as reflected by inhibition of at least one of the biological activities as described in the examples, below. Such screening for molecules that bind Trp8a, Trp8b, Trp9, Trp10a or Trp10b could easily performed on a large scale, e.g. by screening candidate molecules from libraries of synthetic and/or natural molecules. Such an inhibitor is, e.g., a synthetic organic chemical, a natural fermentation product, a substance extracted from a microorganism, plant or animal, or a peptide. Additional examples of inhibitors are specific antibodies, preferably monoclonal antibodies. Moreover, the nucleic sequences of the invention and the encoded proteins can be used to identify further factors involved in tumor development and progression. In this context it should be emphasized that the modulation of the calcium channel of a member of the trp family can result in the stimulation of the immune response of T lymphocytes leading to proliferation of the T lymphocytes. The proteins of the invention can, e.g., be used to identify further (unrelated) proteins which are associated with the tumor using screening methods based on protein/protein interactions, e.g. the two-hybrid-system Fields, S. and Song, O. (1989) Nature (340): 245-246.

The present invention also provides a method for diagnosing a prostate carcinoma which comprises contacting a target sample suspected to contain the protein Trp8a, Trp8b, Trp10a and/or Trp10b or the Trp8a, Trp8b, Trp10a and/or Trp10b encoding mRNA with a reagent which reacts with Trp8a, Trp8b, Trp10a and/or Trp10b or the Trp8a, Trp8b, Trp10a and/or Trp10b encoding mRNA and detecting Trp8a, Trp8b, Trp10a and/or Trp10b or Trp8a, Trp8b, Trp10a and/or Trp 10b encoding mRNA.

It has been found that carcinoma cells of placenta (chorion carcinoma), lung and prostate express Trp10 transcripts as well as Trp10 antisense transcripts and transcripts being in part complementary to Trp10 antisense transcripts. Accordingly, the present invention also provides a method for diagnosing a melanoma, chorion carcinoma, cancer of the lung and of the prostate in a tissue of a subject, comprising contacting a sample with a reagent which detects Trp10a and/or Trp10b antisense RNA.

When the target is mRNA (or antisense RNA), the reagent is typically a nucleic acid probe or a primer for PCR. The person skilled in the art is in a position to design suitable nucleic acids probes based on the information as regards the nucleotide sequence of Trp8a, Trp8b, Trp10a or Trp10b as depicted in figure 7, 8a, 10 and 11, respectively, or tables 1 and 2, above. When the target is the protein, the reagent is typically an antibody probe. The term „antibody", preferably, relates to antibodies which consist essentially of pooled monoclonal antibodies with different epitopic specifities, as well as distinct monoclonal antibody preparations. Monoclonal antibodies are made from an antigen containing fragments of the proteins of the invention by methods well known to those skilled in the art (see, e.g., Köhler et al., Nature 256 (1975), 495). As used herein, the term „antibody" (Ab) or „monoclonal antibody" (Mab) is meant to include intact molecules as well as antibody fragments (such as, for example, Fab and F(ab') 2 fragments) which are capable of specifically binding to protein. Fab and f(ab')2 fragments lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and may have less non-specific tissue binding than an intact antibody. (Wahl et al., J. Nucl. Med. 24: 316-325 (1983)). Thus, these fragments are preferred, as well as the products of a FAB or other immunoglobulin expression library. Moreover, antibodies of the present invention include chimerical, single chain, and humanized antibodies. The target cellular component, i.e. Trp8a, Trp8b, Trp10a and/or Trp10b or Trp8a, Trp8b, Trp10a and/or Trp10b encoding mRNA or Trp10a/b antisense transcripts, e.g., in biological fluids or tissues, may be detected directly in situ, e.g. by in situ hybridization (e.g., according to the examples, below) or it may be isolated from other cell components by common methods known to those skilled in the art before contacting with a probe. Detection methods include Northern blot analysis, RNase protection, in situ methods, e.g. in situ hybridization, in vitro amplification methods (PCR, LCR, QRNA replicase or RNA-transcription/amplification (TAS, 3SR), reverse dot blot disclosed in EP-B1 O 237 362)), immunoassays, Western blot and other detection assays that are known to those skilled in the art.

Products obtained by in vitro amplification can be detected according to established methods, e.g. by separating the products on agarose gels and by subsequent staining with ethidium bromide. Alternatively, the amplified products can be detected by using labeled primers for amplification or labeled dNTPs.

The probes can be detectable labeled, for example, with a radioisotope, a bioluminescent, compound, a chemiluminescent compound, a fluorescent compound, a metal chelate, or an enzyme.

Expression of Trp8a, Trp8b, Trp10a and Trp10b, respectively, in tissues can be studied with classical immunohistological methods (Jalkanen et al., J. Cell. Biol. 101 (1985), 976-985; Jalkanen et al., J. Cell. Biol. 105 (1987), 3087-3096; Sobol et al. Clin. Immunpathol. 24 (1982), 139-144; Sobol et al., Cancer 65 (1985), 2005-2010). Other antibody based methods useful for detecting protein gene expression include immunoassays, such as the enzyme linked immunosorbent assay (ELISA) and the radioimmunoassay (RIA). Suitable antibody assay labels are known in the art and include enzyme labels, such as, glucose oxidase, and radioisotopes, such as iodine (^{125}I, ^{121}I), carbon (^{14}C), sulfur (^{35}S), tritium (^{3}H), indium (^{112}In), and technetium rhodamine, and biotin. In addition to assaying Trp8a, Trp8b, Trp 10a or Trp10b levels in a biological sample, the protein can also be detected in vivo by imaging. Antibody labels or markers for in vivo imaging of protein include those detectable by X-radiography, NMR or ESR. For X-radiography, suitable labels include radioisotopes such as barium or cesium, which emit detectable radiation but are not overtly harmful to the subject. Suitable markers for NMR and ESR include those with a detectable characteristic spin, such as deuterium, which may be incorporated into the antibody by labeling of nutrients for the relevant hybridoma. A protein-specific antibody or antibody fragment which has been labeled with an appropriate detectable imaging moiety, such as a radioisotope (for example, ^{131}I, ^{112}In, ^{99}mTc), a radio-opaque substance, or a material detectable by nuclear magnetic resonance, is introduced (for example, parenterally, subcutaneously, or intraperitoneally) into the mammal. It will be understood in the art that the size of the subject and the imaging system used will determine the quantity of imaging moiety needed to produce diagnostic images. In the case of a radioisotope moiety, for a human subject, the quantity of radioactivity injected will normally range from about 5 to 20 millicuries of ^{99}mTc. The labeled antibody or antibody fragment will then preferentially accumulate at he location of cells which contain the specific protein. In

vivo tumor imaging is described in S.W. Burchiel et al., „Immunopharmacokinetics of Radiolabeled Antibodies and Their Fragments". (Chapter 13 in Tumor Imaging: The Radiochemical Detection of Cancer, S.W. Burchiel and B.A. Rhodes, eds., Masson Publishing Inc. (1982)).

The marker Trp8a and Trp8b is also useful for prognosis, for monitoring the progression of the tumor and the diagnostic evaluation of the degree of malignancy of a prostate tumor (grading and staging), e.g. by using in situ hybridization: In a primary carcinoma Trp8 is expressed in about 2 to 10% of carcinoma cells, in a rezidive carcinoma in about 10 to 60% of cells and in metastases in about 60 to 90% of cells.

The present invention also relates to a method for diagnosing endometrial cancer (cancer of the uterus) which comprises contacting a target sample suspected to contain the protein Trp8a and/or Trp8b or the Trp8a and/or Trp8b encoding mRNA with a reagent which reacts with Trp8a and/or Trp8b or the encoding mRNA and detecting Trp8a and/or Trp8b encoding mRNA. As regards particular embodiments of this method reference is made to the particular embodiments of the method of diagnosing a prostate cancer outlined above.

For evaluating whether the concentration of Trp8a, Trp8b, Trp10a or Trp10b or the concentration of Trp8a, Trp8b, Trp10a or Trp10b encoding mRNA is normal or increased, thus indicative for the presence of a malignant tumor, the measured concentration is compared with the concentration in a normal tissue, preferably by using the ratio of Trp8a:Trp9, Trp8b:Trp9 or Trp10(a or b)/Trp9 for quantification.

Since the prostate carcinoma forms its own basement membrane when growing invasively, it can be concluded that only cells expressing Trp8 and Trp10 are involved in this phenomenon. Thus, it can be concluded that by inhibiting the expression and/or activity of these proteins an effective therapy of cancers like PCA is provided.

Thus, the present invention also relates to a pharmaceutical composition containing a reagent which decreases or inhibits Trp8a, Trp8b, Trp10a and/or Trp10b expression or the activity of Trp8a, Trp8b, Trp10a and/or Trp10b, and a method for preventing, treating, or ameliorating a prostate tumor, endometrial cancer (uterine carcinoma) tumor, a chorion carcinoma, cancer of the lung or melanoma,which comprises administering to a mammalian subject a

therapeutically effective amount of a reagent which decreases or inhibits Trp8a, Trp8b, Trp10a and/or Trp10b expression or the activity of Trp8a, Trp8b, Trp10a and/or Trp10b. Examples of such reagents are the above described antisense RNAs, ribozymes or inhibitors, e.g. specific antibodies. Furthermore, peptides, which inhibit or modulate the biological function of Trp8a, Trp8b, Trp9, Trp10a and/or Trp10b may be useful as therapeutical reagents. For example, these peptides can be obtained by screening combina torial phage display libraries (Cosmix, Braunschweig, Germany) as described by Rottgen, P. and Collins, J. (Gene (1995) 164 (2): 243-250). Furthermore, antigenic epitopes of the Trp8 and Trp10 proteins can be identified by the expression of recombinant Trp8 and Trp10 epitope libraries in E. coli (Marquart, A. & Flockerzi, V., FEBS Lett. 407 (1997), 137-140; Trost, C., et al., FEBS Lett. 451 (1999) 257-263 and the consecutive screening of these libraries with serum of patients with cancer of the prostate or of the endometrium. Those Trp8 and Trp10 epitopes which are immunogenic and which lead to the formation of antibodies in the serum of the patients can be then be used as Trp8 or Trp10 derived peptide vaccines for immune inventions against cancer cells which express Trp8 or Trp10. Alternatively to the E. coli expression system, Trp8 or Trp10 or epitopes of Trp8 and Trp10 can be expressed in mammalian cell lines such as human embryonic kidney (Hek 293) cells (American Type Culture Collection, ATCC CRL 1573).

Finally, compounds useful for therapy of the above described diseases comprise compounds which act as antagonists or agonists on the ion channels Trp8, Trp9 and Trp10. It could be shown that Trp8 is a highly calcium selective ion channel which in the presence of monovalent (namely sodium) and divalent ions (namely calcium) is only permeable for calcium ions (see Example 4, below, and Figures 3A, C, E). Under physiological conditions, Trp8 is a calcium selective channel exhibiting large inward currents. This very large conductance of Trp8 channels (as wells as Trp9 and Trp10a/b channels) is useful to establish systems for screening pharmacological compounds interacting with Trp-channels including high throughput screening systems. Useful high throughput screening systems are well known to the person skilled in the art and include, e.g., the use of cell lines stably or transiently transfected with DNA sequences encoding Trp8, Trp9 and Trp10 channels in assays to detect calcium signaling in biological systems. Such systems include assays based on Ca-sensitive dyes such as aequorin, apoaequorin, Fura-2, Fluo-3 and Indo-1.

Accordingly, the present invention also relates to a method for identifying compounds which act as agonists or antagonists on the ion channels Trp8, Trp9 and/or Trp10, said method comprising contacting a test compound with the ion channel Trp8, Trp9 and/or Trp10, preferably by using a system based on cells stably or transiently transfected with DNA sequences encoding Trp8, Trp9 and/or Trp10, and determining whether said test compound affects the calcium uptake.

For administration the above described reagents are preferably combined with suitable pharmaceutical carriers. Examples of suitable pharmaceutical carriers are well known in the art and include phosphate buffered saline solutions, water, emulsions, such as oil/water emulsions, various types of wetting agents, sterile solutions etc. Such carriers can be formulated by conventional methods and can be administered to the subject at a suitable dose. Administration of the suitable compositions may be effected by different ways, e.g. by intravenous, intraperetoneal, subcutaneous, intramuscular, topical or intradermal administration. The route of administration, of course, depends on the nature of the tumor and the kind of compound contained in the pharmaceutical composition. The dosage regimen will be determined by the attending physician and other clinical factors. As is well known in the medical arts, dosages for any one patient depends on many factors, including the patient's size, body surface area, age, sex, the particular compound to be administered, time and route of administration, the kind and stage of the tumor, general health and other drugs being administered concurrently.

The delivery of the antisense RNAs or ribozymes of the invention can be achieved by direct application or, preferably, by using a recombinant expression vector such as a chimeric virus containing these compounds or a colloidal dispersion system. By delivering these nucleic acids to the desired target, the intracellular expression of Trp8a, Trp8b, Trp10a and/or Trp10b and, thus, the level of Trp8a, Trp8b, Trp10a and/or Trp10b can be decreased resulting in the inhibition of the negative effects of Trp8a, Trp8b, Trp10a and/or Trp10b, e.g. as regards the metastasis formation of PCA.

Direct application to the target site can be performed, e.g., by ballistic delivery, as a colloidal dispersion system or by catheter to a site in artery. The colloidal dispersion systems which can be used for delivery of the above nucleic, acids include macromolecule complexes, nanocapsules, microspheres, beads and lipid-based systems including oil-in-water emulsions

(mixed), micelles, liposomes and lipoplexes, The preferred colloidal system is a liposome. The composition of the liposome is usually a combination of phospholipids and steroids, especially cholesterol. The skilled person is in a position to select such liposomes which are suitable for the delivery of the desired nucleic acid molecule. Organ-specific or cell-specific liposomes can be used in order to achieve delivery only to the desired tumor. The targeting of liposomes can be carried out by the person skilled in the art by applying commonly known methods. This targeting includes passive targeting (utilizing the natural tendency of the liposomes to distribute to cells of the RES in organs which contain sinusoidal capillaries) or active targeting (for example by coupling the liposome to a specific ligand, e.g., an antibody, a receptor, sugar, glycolipid, protein etc., by well known methods). In the present invention monoclonal antibodies are preferably used to target liposomes to specific tumors via specific cell-surface ligands.

Preferred recombinant vectors useful for gene therapy are viral vectors, e.g. adenovirus, herpes virus, vaccinia, or, more preferably, an RNA virus such as a Retrovirus. Even more preferably, the retroviral vector is a derivative of a murine or avian retrovirus. Examples of such retroviral vectors which can be used in the present invention are: Moloney murine leukemia virus (MoMuLV), Harvey murine sarcoma virus (HaMuSV), murine mammary tumor virus (MuMTV) and Rous sarcoma virus (RSV). Most preferably, a non-human primate retroviral vector is employed, such as the gibbon ape leukemia virus (GaLV), providing a broader host range compared to murine vectors. Since recombinant retroviruses are defective, assistance is required in order to produce infectious particles. Such assistance can be provided, e.g., by using helper cell lines that contain plasmids encoding all of the structural genes of the retrovirus under the control of regulatory sequences within the LTR. Suitable helper cell lines are well known to those skilled in the art. Said vectors can additionally contain a gene encoding a selectable marker so that the transduced cells can be identified. Moreover, the retroviral vectors can be modified in such a way that they become target specific. This can be achieved, e.g., by inserting a polynucleotide encoding a sugar, a glycolipid, or a protein, preferably an antibody. Those skilled in the art know additional methods for generating target specific vectors. Further suitable vectors and methods for in vitro- or in vivo-gene therapy are described in the literature and are known to the persons skilled in the art; see, e.g., WO 94/29469 or WO 97/00957.

In order to achieve expression only in the target organ, i.e. tumor to be treated, the nucleic acids encoding, e.g. an antisense RNA or ribozyme can also be operably linked to a tissue specific promoter and used for gene therapy. Such promoters are well known to those skilled in the art (see e.g. Zimmermann et al., (1994) Neuron 12, 11-24; Vidal et al.; (1990) EMBO J. 9, 833-840; Mayford et al., (1995), Cell 81, 891-904; Pinkert et al., (1987) Genes & Dev. 1, 268-76).

For use in the diagnostic research discussed above, kits are also provided by the present invention. Such kits are useful for the detection of a target cellular component, which is Trp8a, Trp8b, Trp10a and/or Trp10b or, alternatively, Trp8a, Trp8b, Trp10a and/or Trp10b encoding mRNA or Trp10a/b antisense transcripts, wherein the presence or an increased concentration of Trp8a, Trp8b, Trp10a and/or Trp10b or, alternatively, Trp8a, Trp8b, Trp10a and/or Trp10b encoding mRNA or Trp10a/b antisense transcripts is indicative for a prostate tumor, endometrial cancer, melanoma, chorion carcinoma or cancer of the lung, said kit comprising a probe for detection of Trp8a, Trp8b, Trp9, Trp10a and/or Trp10b or, alternatively, Trp8a, Trp8b, Trp9, Trp10a and/or Trp10b encoding mRNA or Trp10a/b antisense transcripts. The probe can be detectably labeled. Such probe may be a specific antibody or specific oligonucleotide. In a preferred embodiment, said kit contains an anti-Trp8a-, anti-Trp8b-, anti-Trp9-, anti-Trp10a-and/or anti-Trp10b-antibody and allows said diagnosis, e.g., by ELISA and contains the antibody bound to a solid support, for example, a polystyrene microtiter dish or nitrocellulose paper, using techniques known in the art. Alternatively, said kits are based on a RIA and contain said antibody marked with a radioactive isotope. In a preferred embodiment of the kit of the invention the antibody is labeled with enzymes, fluorescent compounds, luminescent compounds, ferromagnetic probes or radioactive compounds. The kit of the invention may comprise one or more containers filled with, for example, one or more probes of the invention. Associated with container (s) of the kit can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, us or sale for human administration.

EXAMPLES

The following Examples are intended to illustrate, but not to limit the invention. While such Examples are typical of those that might be used, other methods known to those skilled in the art may alternatively be utilized.

Example 1: Materials and Methods

(A) Isolation of cDNA clones and Northern blot analysis

Total RNA was isolated from human placenta an prostate using standard techniques. Isolation of mRNA was performed with poly $(A)^+$RNA - spin columns (New England Biolabs, Beverly, USA) according to the instructions of the manufacturer. Poly (a) $^+$RNA was reverse transcribed using the cDNA choice system (Gibco-BRL, Rockville, USA) and subcloned in λ-Zap phages (Stratagene, La Jolla, USA). An human expressed sequence tag (GenBank accession number 1404042) was used to screen an oligo d(T) primed human placenta cDNA library. Several cDNA clones were identified and isolated. Additional cDNA clones were isolated from two specifically primed cDNA libraries using primers 5'-gca tag gaa ggg aca ggt gg-3' and 5'-gag agt cga ggt cag tgg tcc-3'.

cDNA clones were sequenced using a thermocycler (PE Applied Biosystems, USA) and Thermo Sequenase (Amersham Pharmacia Biotech Europe, Freiburg, Germany). DNA sequences were analyzed with an automated sequencer (Licor, Linccoln, USA).

For Northern blot analysis 5 µg human poly $(A)^+$ RNA from human placenta or prostate were separated by electrophoresis on 0.8 % agarose gels. Poly $(A)^+$ RNA was transferred to Hybond N nylon membranes (Amersham Pharmacia Biotech Europe, Freiburg, Germany). The membranes were hyridized in the presence of 50 % formamide at 42^0C over night. DNA probes were labelled using [α^{32}P]dCTP and the „ready prime„ labelling kit (Amersham Pharmacia Biotech Europe, Freiburg, Germany). Commercial Northern blots were hybridized according to the distributors instructions (Clontech, Paolo Alto, USA).

(B) Construction of expression plasmids and transfection of HEK 293 cells

Lipofections were carried out with the recombinant dicistronic eucaryotic expression plasmid pdiTRP8 containing the cDNA of Trp8b under the control of the chicken ß-actin promotor followed by an internal ribosome entry side (IRES) and the cDNA of the green fluorescent protein (GFP). To obtain pdiTRP8 carrying the entire protein coding regions of TRP8b and

the GFP (Prasher, D.C. et al. (1992), Gene 111, 229-233), the 5`and 3´-untranslated sequences of the TRP8b cDNA were removed, the consensus sequence for initiation of translation in vertebrates (Kozak, M. (1987) Nucleic Acids Research15, 8125-8148) was introduced immediately 5`of the translation initiation codon and the resulting cDNA was subcloned into the pCAGGS vector (Niwa, H., Yamamura, K. and Miyazaki, J (1991), Gene 8, 193-199) downstream of the chicken β-actin promotor. The IRES derived from encephalmyocarditis virus (Kim, D.G., Kang, H.M., Jang, S.K. and Shin H.S. (1992) Mol.Cell.Biol. 12, 3636-3643) followed by the GFP cDNA containing a Ser65Thr mutation (Heim, R., Cubitt, A.B., Tsien, R.Y. (1995) Nature 373, 663-664) was then cloned 3` to the TRP8b cDNA. The IRES sequence allows the simultaneous translation of TRP8b and GFP from one transcript. Thus, transfected cells can be detected unequivocally by the development of green fluorescence.

For monitoring of the intracellular Ca^{2+} concentration human embryonic kidney (HEK 293) cells were cotransfected with the pcDNA3-TRP8b vector and the pcDNA3-GFPvector in a molar ratio of 4 : 1 in the presence of lipofectamine (Quiagen, Hilden, Germany). To obtain pcDNA3-TRP8b the entire protein coding region of TRP8b including the consensus sequence for initiation of translation in vertebrates (Kozak, M. (1987) Nucleic Acids Research15, 8125-8148) was subcloned into the pcDNA3 vector (Invitrogen, Groningen, Netherlands). Calcium monitoring and patch clamp experiments were carried out two days and one day after transfection, respectively.

(C) Chromosomal localization of the Trp8 gene

The chromosomal localization of the human TRP8 gene was performed using NIGMS somatic hybrid mapping panel No.2 (Coriell Institute, Camden, NJ, USA) previously described (Drwinga, H.L., Toji, L.H., Kim, C.H., Greene, A.E., Mulivor, R.A. (1993) Genomics 16, 311-314; Dubois, B.L. and Naylor, S.L. (1993) Genomics 16, 315-319).

(D) In Vitro Translation, glutathione - sepharose and calmodulin agarose binding assay

N– and C-terminal Trp8-fragments were subcloned into the pGEX-4T2 vector (Amersham Pharmacia Europe, Freiburg, Germany) resulting in glutathione-S-transferase (GST)-Trp8 fusion constructs (Fig. 4). The GST-TRP8-fusion proteins were expressed in E. coli BL 21 cells and purified using glutathione - sepharose beads (Amersham Pharmacia Biotech Europe, Freiburg, Germany).

In vitro translation of human Trp8 cDNA and Xenopus laevis calmodulin cDNA (Davis, T.N. and Thorner, J. Proc.Natl.Acad.Sci. USA 86, 7909-7913.) was performed in the presence of ^{35}S-methionine using the TNT coupled transcription/translation kit (Promega, Madison, USA). Translation products were purified by gel fliltration (Sephadex G50, Amersham Pharmacia Biotech Europe, Freiburg, Germany) and equal amounts of ^{35}S labeled probes were incubated for 2 h with glutathione beads bound to GST - Trp8 or calmodulin - agarose (Calbiochem) in 50 mM Tris-HCl, pH 7.4, 0.1 % Triton X-100, 150 mM NaCl in the presence of 1 mM Ca^{2+} or 2 mM EGTA. After three washes, bound proteins were eluted with SDS sample buffer, fractionated by SDS-PAGE and ^{35}S labeled proteins were detected using a Phosphor Imager (Fujifilm, Tokyo, Japan).

(E) Calcium measurements

The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was determined by dual wavelength fura-2 fluorescence ratio measurements (Tsien, R.Y. (1988) Trends Neurosci. 11, 419-424) using a digital imaging system (T.I.L.L. Photonics, Planegg, Germany). HEK cells were grown in minimal essential medium in the presence of 10 % fetal calf serum and cotransfected with the pcDNA3-TRP8b vector and the pCDNA3-GFPvector as described above (B). Transfected cells were detected by development of green fluorescence. The cells were loaded with 4μM fura-2/AM (Molecular Probes, Oregon, USA) for one hour. After loading the cells were rinsed 3 times with buffer B1 (10 mM Hepes, 115 mM NaCl, 2 mM $MgCl_2$, 5mM KCl, pH 7.4) and the $[Ca^{2+}]_i$ was calculated from the fluorescence ratios obtained at 340 and 380 nm excitation wavelengths as described (Garcia, D.E., Cavalié, A. and Lux, H.D. (1994) J. Neurosci 14, 545-553).

(F) Electrophysiological recordings

HEK cells were transfected with the eucaryotic expression plasmid pdiTRP8 described in (B) and electrophysiolocigal recordings were carried out one day after transfection. Single cells were voltage clamped in the whole cell mode of the patch clamp technique as described (Hamill, O.P., Marty, A., Neher, E., Sakmann, B. and Sigworth, F.J. (1981) Pflügers Arch. 391, 85-100; Philipp, S., Cavalié, A., Freichel, M., Wissenbach, U., Zimmer, S., Trost, C., Marquart, A., Murakami, M. and Flockerzi, V. (1996) EMBO J. 6166-6171). The pipette solution contained contained (mM): 140 aspartic acid, 10 EGTA, 10 NaCl, 1 MgCl2, 10 Hepes (pH 7.2 with CsOH) or 125 CsCl, 10 EGTA, 4 $CaCl_2$ 10 Hepes (pH 7,2 with CsOH). The bath solution contained (mM): 100 NaCl, 10 CsCl, 2 $MgCl_2$, 50 mannitol, 10 glucose, 20

Hepes (pH 7,4 with CsOH) and 2 $CaCl_2$, or no added $CaCl_2$ (-Ca^{2+} solution). Divalent free bath solution contained (mM): 110 N-methyl-D-glucamine (NMDG). Whole cell currents were recorded during 100 msec voltage ramps from –100 to +100 mV at varying holding potentials.

(G) In Situ Hybridization

In situ hybridizations were carried out using formalin fixed tissue slices of 6 - 8 µM thickness. The slices were hydrated and incubated in the presence of PBS buffer including 10 µg / ml proteinase K (Roche Diagnostics, Mannheim, Germany) for 0.5 h. The slices were hybridized at 37°C using biotinylated deoxy-oligonucleotides (0.5 pmol / µl) in the presence of 33 % formamide for 12 h. Furthermore the slices were several times rinsed with 2 x SSC and incubated at 25°C for 0.5 h with avidin / biotinylated horse raddish peroxidase complex (ABC, DAKO, Santa Barbara, USA). After several washes with PBS buffer the slices were incubated in the presence of biotinylated tyramid and peroxide (0.15 % w/v) for 10 min, rinsed with PBS buffer and additionally incubated with ABC complex for 0.5 h. The slices were washed with PBS buffer and incubated in the presence of DAB solution (diaminobenzidine (50µg / ml), 50 mM Tris/EDTA buffer pH 8.4, 0.15 % H_2O_2 in N,N - dimethyl-formamide; Merck, Darmstadt, Germany), The detection was stopped after 4 minutes by incubating the slides in water. Tyramid was biotinylated by incubating NHS-LC Biotin (sulfosuccinimidyl-6-(biotinimid)-hexanoat), 2.5 mg / ml; Pierce, Rockford, USA) and tyramin-HCl (0.75 mg / ml, Sigma) in 25 mM borate buffer pH 8.5 for 12 h. The tyramid solution was diluted 1 - 5 : 1000 in PBS buffer.

(H) GenBank accession numbers: TRP8a, Aj243500; TRP8b Aj243501

Example 2: Expression of TRP8 transcripts

In search of proteins distantly related to the TRP family of ion channels, an human expressed sequence tag (EST, GenBank accession number 1404042) was identified in the GenBank database using BLAST programms (at the National Center for Biotechnology Information (NCBI); Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J.J. (1990) Mol. Biol. 5, 403-410) being slightly homologous to the VR1 gene. Several human placenta cDNA libraries were constructed and screeened with this EST DNA as probe. Several full length

cDNA clones were identified and isolated. The full length cDNA clones encoded two putative proteins differing in three amino acids and were termed Trp8a and Trp8b (Fig. 1c, 2a, 7 and 8A). This finding was reproduced by isolating cDNA clones from two cDNA libraries constructed from two individual placentas. The derived protein sequence(s) comprises six transmembrane domains, a characteristic overall feature of trp channels and related proteins (Fig.: 1b). The sequence is closely related to the meanwhile published calcium uptake transport protein 1 (CaT1), isolated from rat intestine (Peng, J.B., Chen, X.Z., Berger, U.V., Vassilev, P.M., Tsukaguchi, H., Brown, E.M. and Hediger M.A.(1999) J Biol Chem. 6;274, 22739-22746) and to the epithelial calcium uptake channel (ECaC) isolated from rabbit kidney (Hoenderop, J.G., van der Kemp, A.W., Hartog, A., van de Graaf, S.F., van Os, C.H., Willems, P.H. and Bindels, R.J. (1999) J Biol Chem. 26;274, 8375-8378). Expression of Trp8a/b transcripts are detectable in human placenta, pancreas and prostate (Fig.: 5) and the size of the Northern signal (3.0 kb) corresponds with the size of the isolated full length cDNAs. In addition, a shorter transcript of 1.8 kb, probably a splice variant, is detectable in human testis. The Trp8 mRNA is not expressed in small intestine or colon (Fig.: 5) implicating that Trp8 is not the human ortholog of the rat CaT1 or rabbit ECaC proteins. To investigate whether there are other related sequences Trp8a/b derived primers (UW241, 5'-TAT GAG GGT TCA GAC TGC-3' and UW242, 5'-CAA AGT AGA TGA GGT TGC-3') were used to amplify a 105 bp fragment from human genomic DNA being 95% identical on the nucleotide level to the Trp8 sequence (data not shown). This indicates the existence of several similar sequences in humans at least at the genomic level.

Example 3: Two variants of the Trp8 protein (Trp8a and Trp8b) arise by polymorphism

Two variants of the Trp8 cDNA were isolated from human placenta (Fig.: 2A, 7 and 8A) which encoded two proteins which differ in three amino acids and were termed Trp8a and Trp8b. Trp8a/b specific primers were designed to amplify a DNA fragment of 458 bp of the Trp8 gene from genomic DNA isolated from human T-lymphocytes (primer pair: UW243, 5'-CAC CAT GTG CTG CAT CTA CC-3' and UW244, 5'-CAA TGA CAG TCA CCA GCT CC-3'). The amplification product contains a part of the sequence where the derived protein sequence of Trp8a comprises the amino acid valine and the Trp8b sequence methionine as well as a silent base pair exchange (g versus a) and an intron of 303bp (Fig.: 2.A, B). Both variants of the Trp8 genes (a,b) were amplified from genomic DNA in equal amounts indicating the existence of both variants in the human genome and therefore being not the

result of RNA editing (Fig.: 2B). The Trp8a gene can be distinguished from the Trp8b gene by cutting the genomic fragment of 458bp with the restriction enzyme Bsp1286I (Fig. 2B). Using human genomic DNA isolated from blood of twelve human subjects as template, the 458bp fragment was amplified and restricted with BSP1286I. In eleven of the tested subjects only the Trp8b gene is detectable, while one subject (7) contains Trp8a and Trp8b genes (Fig.: 2D). These implicates that the two Trp8 variants arise by polymorphism and do not represent individual genes. Using Trp8 specific primers and chromosomal DNA as template, the Trp8 locus is detectable on chromosome 7 (Fig.: 2C).

Example 4: Trp8b is a calcium permeable channel

The protein coding sequence of the Trp8b cDNA was subcloned into pcDNA3 vector (Invitrogen, Groningen, Netherlands) under the control of the cytomegalovirus promotor (CMV). Human embryonic kidney (HEK 293) cells were cotransfected with the Trp8b pcDNA3 construct (pcDNA3-Trp8b vector) and the pcDNA3-GFPvector encoding the green fluorescent protein (GFP) in 4:1 ratio. The Trp8b cDNA and the cDNA of the reporter, GFP, was transiently expressed in human embryonic kidney (HEK 293) cells. The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and changes of $[Ca^{2+}]_i$ were determined by dual wavelength fura-2 fluorescence ratio measurements (Fig.: 3F) in cotransfected cells which were identified by the green fluorescence of the reporter gene GFP.

Dual wavelength fura-2 fluorescence ratio measurement is a standard procedure (e.g. in: An introduction of Molecular Neurobiology (ed. Hall, Z.W.)Sinauer Associates, Sunderland, USA (1992)) using fura-2, which is a fluorescent Ca^{2+} sensitive dye and which was designed by R.Y.Tsien (e.g. Trends Neurosci. 11, 419-424 (1988) based upon the structure of EGTA. Its fluorescence emission spectrum is altered by binding to Ca^{2+} in the physiological concentration range. In the absence of Ca^{2+}, fura-2 fluoresces most strongly at an excitation wavelength of 385 nm; when it binds Ca^{2+}, the most effective excitation wavelength shifts to 345 nm. This property is used to measure local Ca^{2+} concentrations within cells. Cells can be loaded with fura-2 esters (e.g. fura-2AM) that diffuse across cell membranes and are hydrolyzed to active fura-2 by cytosolic esterases.

In the presence of 1mM Ca^{2+}, Trp8 expressing cells typically contained more than 300 nM cytosolic Ca^{2+}, while non transfected controls contained less than 100 nM Ca^{2+} ions (Fig. 3F).

When Trp8b transfected cells were incubated without extracellular Ca^{2+}, the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) decreased to levels comparable to non transfected cells. Readdition of 1mM Ca^{2+} to the bath resulted in significant increase of the cytosolic $[Ca^{2+}]$ in Trp8b transfected cells, but not in controls (Fig.: 3F). After readdition of Ca^{2+} ions to the bath solution, the cytosolic Ca^{2+} concentration remains on a high steady state level in the Trp8b transfected cells.

Example 5: Trp8 expressing cells show calcium selective inward currents

To characterize in detail the electrophysiological properties of TRP8, TRP8 and GFP were coexpressed in HEK293 cells using the dicistronic expression vector pdiTRP8 and measured currents using the patch clamp technique in the whole cell mode (Hamill, O.P., Marty, A., Neher, E., Sakmann, B. and Sigworth, F.J. (1981) Pflugers Arch., 391, 85-100).

The eucaryotic expression plasmid pdiTRP8 contains the cDNA of Trp8b under the control of the chicken ß-actin promotor followed by an internal ribosome entry side (IRES) and the cDNA of the green fluorescent protein (GFP). To obtain pdiTRP8 carrying the entire protein coding regions of TRP8b and the GFP (Prasher, D.C. et al. (1992), Gene 111, 229-233), the 5`and 3´-untranslated sequences of the TRP8b cDNA were removed, the consensus sequence for initiation of translation in vertebrates (Kozak, M. (1987) Nucleic Acids Research15, 8125-8148) was introduced immediately 5`of the translation initiation codon and the resulting cDNA was subcloned into the pCAGGS vector (Niwa, H., Yamamura, K. and Miyazaki, J (1991), Gene 8, 193-199) downstream of the chicken β-actin promotor. The IRES derived from encephalmyocarditis virus (Kim, D.G., Kang, H.M., Jang, S.K. and Shin H.S. (1992) Mol.Cell.Biol. 12, 3636-3643) followed by the GFP cDNA containing a Ser65Thr mutation (Heim, R., Cubitt, A.B., Tsien, R.Y. (1995) Nature 373, 663-664) was then cloned 3` to the TRP8b cDNA. The IRES sequence allows the simultaneous translation of TRP8b and GFP from one transcript. Thus, transfected cells can be detected unequivocally by the development of green fluorescence.

In the presence of 2 mM external calcium, Trp8b transfected HEK cells show inwardly rectifying currents, the size of which depends on the level of intracellular calcium and the electrochemical driving force. The resting membrane potential was held either at –40 mV, or, to lower the driving force for calcium influx in between pulses, at + 70 mV. Current traces

were recorded in response to voltage ramps from –100 to +100 mV, that were applied every second. To monitor inward and outward currents over time, we analyzed the current size at – 80 and + 80 mV of the ramps. Figure 3A shows a representative trace of the current at – 80 mV over time. Both at a holding potential of –40 mV or at +70 mV, the currents are significantly larger than in cells transfected with only the GFP containing vector (Fig.: 3E). Interestingly, after changing to a positive holding potential, current size in Trp8 transfected cells slowly increases and reaches steady state after approximately 70 seconds (Fig.: 3A). To determine the selectivity of the induced currents, we then perfused the cells with solutions that either contain no sodium, no added Ca^{2+} (Fig. 3A, C) or a sodium containing, but divalent ion free bath solution. To control for the effect of the solution change alone, we also perfused with normal bath (see puff in Fig. 3A). While removal of external Ca^{2+} completely abolishes the trp 8 induced currents - the remaining current being identical in size and shape to the control (Fig.: 3A, C, E), removal of external sodium has no effect (Fig.: 3E). An important hallmark of calcium selective channels (e.g. Vennekens, R., Hoenderop, G.J., Prenen, J., Stuiover, M., Willems, PHGM, Droogmans, G., Nilius, B.and Bindels, R.J.M (1999) J. Biol. Chem. 275, 3963-3969), is their ability to conduct sodium only if all external divalent ions, namely Ca^{2+} and magnesium are removed. To test whether the trp 8 channel conforms with this phenomenon normal bath solution was switched to a solution containing only sodium and 1 mM EGTA. As can be seen in Figure 3B and D, Trp8 transfected cells can now conduct very large sodium currents. Interestingly, immediately after the solution change, the currents first become smaller before increasing rapidly, indicating that the pore may initially still be blocked by calcium a phenomenon usually called anomalous mole fraction behaviour (Warnat, J., Philipp, S., Zimmer, S.,.Flockerzi, V., and Cavalié A.(1999) J.Physiol. (Lond) 518, 631-638). The measured outward currents of Trp8 transfected cells in normal bath solution are not significantly different from non-transfected control cells or cells which only express the reporter gene GFP. As the removal of external Ca^{2+} abolishes the Trp8 specific current, the remaining current was subtracted from the current before the solution change to obtain the uncontaminated Trp8 conductance (see inset in Fig.: 3C). As expected from the given ionic conditions (high EGTA inside, 2 mM Ca^{2+} outside), the current-voltage relationship now shows prominent inward rectification with little to no outward current.

Both the time course of the development of Trp8 currents and the size of the currents depend on the frequency of stimulation (data not shown), the internal and external Ca^{2+} concentration

and the resting membrane potential, suggesting that Trp8 calcium conductance is intrically regulated by a Ca^{2+} mediated feedback mechanisms.

Example 6: Ca^{2+} / calmodulin binds to the C-terminus of the Trp8 protein

To test whether calmodulin, a prime mediator of calcium regulated feedback, is involved, first it was investigated biochemically whether Trp8 protein can bind calmodulin. Trp8 cDNA was in vitro translated in the presence of ^{35}S-methionine and the product incubated with calmodulin-agarose beads. After several washes either in the presence or abscence of Ca^{2+}, the beads were incubated in Laemmli buffer and subjected to SDS-polyacrylamide gel electrophoresis. In the presence of Ca^{2+} (1mM), but not in the absence of Ca^{2+}, Trp8 protein binds to calmodulin (Fig.: 4B).

To narrow down the binding site, two approaches were undertaken: Firstly, GST-TRP8 fusion proteins of various intracellular domains of Trp8 were constructed, expressed in E. coli and bound to gluthathione sepharose beads. These beads were then incubated with in vitro translated ^{35}S- labeled calmodulin, washed and subjected to gel electrophoresis. Secondly, truncated versions of in vitro translated Trp8 protein were used in the above described binding to calmodulin-agarose. As shown in Figure 4A, and C, fusion proteins of the N-terminal region (N1, N2) of Trp8 did not bind calmodulin, while C-terminal fragments (C1, C2, C3, C4) showed calmodulin binding in the presence of calcium (for localization of fragments within the entire Trp8 protein see Fig. 4C). Accordingly, a truncated version of in vitro translated Trp8, which lacks the C-terminal 32 amino acid residues did not bind to calmodulin-agarose (4B). We have restricted the calmodulin binding site to amino acid residues 691 to 711 of the Trp8 protein. This calmodulin binding site does not resemble the typical conserved IQ - motif of conventional myosins, but has limited sequence homology to the calcium dependent calmodulin binding site 1 of the transient receptor potential like (trpl) protein of Drosophila melanogaster (Warr and Kelly, 1996) with several charged amino acid residues conserved. The sequence of the calmodulin binding site of the Trp8 protein resembles a putative amphipathic α-helical wheel structure with a charged and a hydrophobic site according to a model proposed by Erickson-Vitanen and De Grado (1987, Methods Enzymol. 139, 455-478.).

Example 7: Expression of Trp8 transcripts in human placenta and pancreas

Several slides from a human placenta of a ten week old abort were used for in situ hybridization experiments. The in situ hybridization experiments revealed expression of Trp8 transcripts in human placenta (Fig.: 5B). Expression was detectable in trophoblasts and syncytiotrophoblasts of the placenta, but not in Langhans cells.

Trp8 transcripts are detectable in human pancreas (Fig.: 5A). Therefore Trp8 probes were hybridized to tissue sections of human pancreas. The pancreatic tissues were removed from patients with pancreas cancer. Trp8 expression is detectable in pancreatic acinar cells, but not in Langerhans islets (Fig.: 5C). No Trp8 expression was found in regions of pancreatic carcinomas (data not shown).

Furthermore, the Trp8 cDNA is not detectable in human colon nor in human kidney by in situ hybridization as well as by Northern analysis (Fig.: 5A, D). The Northern results taken together with the in situ expression data indicate that the Trp8 protein is not the human ortholog of the CaT1 and ECaC channels cloned from rat intestine (Peng, J.B., Chen, X.Z., Berger, U.V., Vassilev, P.M., Tsukaguchi, H., Brown, E.M. and Hediger M.A.(1999) J Biol Chem. 6;274, 22739-22746) and from rabbit kidney (Hoenderop, J.G., van der Kemp, A.W., Hartog, A., van de Graaf, S.F., van Os, C.H., Willems, P.H. and Bindels, R.J. (1999) J Biol Chem. 26;274, 8375-8378), respectively. Trp8 is unlikely to represent the human version of CaT1 as its expression is undetectable in the small intestine and colon tissues where CaT1 is abundantly expressed. If, however, Trp8 is the human version of rat CaT1, a second gene product appears to be required for Ca^{2+} uptake in human small intestine and colon attributed to CaT1 in rat small intestine and colon.

Example 8: Differential expression of Trp8 transcripts in benign and malign tissue of the prostate

The Trp8 transcripts are expressed in human prostate as shown by hybridization of a Trp8 probe to a commercial Northern blot (Clontech, Palo Alto, USA) (Fig.: 5A). Trp8 transcripts were not detectable by Northern blot analysis using pooled mRNA of patients with benign prostatic hyperplasia (BPH) (Fig.: 5A, prostate*). To examine Trp8 expression on the cellular

level, sections of prostate tissues were hybridized using Trp8 specific cDNA probes (Table 3). Expression of Trp8 transcripts is not detectable in normal prostate (n = 3), benign hyperplasia (BPH, n = 15) or prostatic intraepithelial neoplasia (PIN, n = 9) (Fig.: 6A, C, E). Trp8 transcripts were only detectable in prostate carcinoma (PCA), although with different expression levels. Low expression levels were found in primary carcinomas (2 - 10 % of the carcinoma cells, n = 8) (Fig.: 7B) . Much stronger expression was detectable in rezidive carcinoma (10 - 60 %) (Fig.: 7D, n = 6) and metastases of the prostate (60 - 90 %, n = 4) (Fig.: 7F). Thus it has to be concluded that the commercial Northern blot used in Fig.: 5A contains not only normal prostate mRNA as indicated by the distributor. According to the distributors instructions the prostate mRNA used for this Northern blot was collected from 15 human subjects in the range of 14 to 60 years of age. This prostate tissue was not examined by pathologic means. Since Trp8 expression is not detectable in normal or benign prostate, this finding implicates that the mRNA used for this Northern blot was extracted in part from prostatic carcinoma tissue. To summarize, Trp8 expression is only detectable in malign prostate and, thus, the Trp8 cDNA is a marker for prostate carcinoma. The results are summarized in Table 4.

Table 3

Trp8 probes used for in situ hybridization:

Probes (antisense)

1.) 5' TCCGCTGCCGGTTGAGATCTTGCC 3'

2.) 5' CTTGCTCCATAGGCAGAGAATTAG 3'

3.) 5' ATCCTCAGAGCCCCGGGTGTGGAA3'

Controls (sense)

1.) 5' GGCAAGATCTCAACCGGCAGCGGA 3'

2.) 5' CTAATTCTCTGCCTATGGAGCAAG 3'

3.) 5' TTCCACACCCGGGGCTCTGAGGAT 3'

Table 4

Prostate	total	negative	positive
normal	3	3	0
BPH	15	15	0
PIN	9	9	0

carcinoma	18	1	17

(B) Differential expression of Trp8 transcripts in benign and malign tissue of the uterus

Moreover it could be shown that Trp8 is expresed in endometrial cancer (also called cancer of the uterus, to be distinguished from uterine sarcoma or cancer of the cervix) whereas no expression was observed in normal uterus tissue. Thus, Trp8 also is a specific marker for the diagnosis of the above cancer (Fig. 12).

Example 9: Characterization of Trp9

The complete protein coding sequence of Trp9 was determined (Fig. 9). Trp 9 transcripts are predominantly expressed in the human prostate and in human colon. As it could be shown by Northern blot analysis, there is no difference of the expression of TRP9 in benigne prostata hyperplasia (BPH, Fig. 13, upper panel left) or prostate carcinoma (Fig. 13, upper panel right). However, Trp9 is useful as a reference marker for prostata carcinoma, i.e. can be used for quantifying the expression level of Trp8. The ratio of the expression of Trp8:Trp9 in patients and healthy individuals is useful for the development of a quantitative assay.

Example 10: Characterization of Trp10

The complete protein coding sequence of TRP10 (a and b) was determined by biocomputing (Fig. 10 and 11). Using a 235 bp fragment of the Trp10 cDNA as probe in Northern blot analysis TRP10 transcripts could only be detected in mRNA isolated from individuals with prostate cancer (Fig. 13, bottom panel) but not in mRNA isolated from benign tissue of the prostate (prostate BPH) nor in mRNA isolated from heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas. The 235 bp cDNA fragment of the Trp10 cDNA was amplified using the primer pair UW248 5'-ACA GCT GCT GGT CTA TTC C-3' and UW249 5'-TAT

GTG CCT TGG TTT GTA CC-3' and prostate cDNA as template. In summary, Trp10a and Trp10b , like TRP8 are also expressed in malignant prostate tissue. So far, its expression could not be observed in any other tissue examined (see above). Thus, Trp 10a and Trp10b are also useful markers which are specific for malignant prostate tissue.

Furthermore, database searches in public databases of the national center for biological information (NCBI) revealed the existence of several expressed sequence tags (EST clones) being in part identical to the Trp10 sequence. These EST clones were originally isolated from cancer tissues of lung, placenta, prostate and from melanoma. These clones include the clones with the following accession numbers: BE274448, BE408880, BE207083, BE791173, AI671853, BE390627. The results demonstrate that cancer cells of these tissues express Trp10 related transcripts whereas no expression of Trp10 transcripts in the corresponding healthy tissues are detectable (Figure 13). Furthermore, it could be shown that in cancer cells of melanoma and prostate cancer Trp10 transcripts are expressed as shown by in situ hybridizations using 4 antisense probes (Figure 14A – E and 13K-O and Table 2, above). Furthermore, it could clearly be shown that cancer cells of these tissues expressing Trp10 transcripts also express Trp10-antisense transcripts as shown in Figure 14F-J, Figure 14P-R and Figure14T by in situ hybridizations using 4 sense probes (Table 2, above). The in situ hybridization experiments demonstrate that detection of a subset of cancer cells derived from carcinoma of lung, placenta, prostate and melanoma is feasible using antisense as well as sense probes complementary to Trp10 transcripts or complementary to Trp10-antisense transcripts, respectively.

The foregoing is meant to illustrate but not to limit the scope of the invention. The person skilled in the art can readily envision and produce further embodiment, based on the above teachings, without undue experimentation.

What Is claimed Is:

1. An isolated nucleic acid molecule encoding the human prostate carcinoma associated protein Trp8a, Trp8b, Trp9, Trp10a or Trp10b or a protein exhibiting biological properties of Trp8a, Trp8b, Trp9, Trp10a or Trp10b and being selected from the group consisting of

(a) a nucleic acid molecule encoding a protein that comprises the amino acid sequence depicted in Figure 7, 8A, 9, 10 or 11;

(b) a nucleic acid molecule comprising the nucleotide sequence depicted in Figure 7, 8A, 9, 10 or 11;

(c) a nucleic acid molecule included in DSMZ Deposit No. DSM 13579, DSM 13580, DSM 13584 , DSM 13581 or DSM....;

(d) a nucleic acid molecule which hybridizes to a nucleic acid molecule specified in (a) to (c);

(e) a nucleic acid molecule the nucleic acid sequence of which deviates from the nucleic sequences specified in (a) to (d) due to the degeneration of the genetic code; and

(f) a nucleic acid molecule, which represents a fragment, derivative or allelic variation of a nucleic acid sequence specified in (a) to (e).

2. A recombinant vector containing the nucleic acid molecule of claim 1

3. The recombinant vector of claim 2 wherein the nucleic acid molecule is operatively linked to regulatory elements allowing transcription and synthesis of a translatable RNA in prokaryotic and/or eukaryotic host cells.

4. A recombinant host cell which contains the recombinant vector of claim 3.

5. The recombinant host cell of claim 4, which is a mammalian cell, a bacterial cell, an insect cell or a yeast cell.

6. An isolated protein exhibiting biological properties of the human prostate carcinoma associated protein Trp8a, Trp8b, Trp9, Trp10a or Trp10b which is encoded by a nucleic acid molecule of claim 1.

7. A recombinant host cell that expresses the isolated protein of claim 6.

8. A method of making an isolated protein exhibiting biological properties of the human prostate carcinoma associated protein Trp8a, Trp8b, Trp9, Trp10a or Trp10b comprising:
(a) culturing the recombinant host cell of claim 6 under conditions such that said protein is expressed; and
(b) recovering said protein.

9. The protein produced by the method of claim 8.

10. An antisense RNA sequence characterized in that it is complementary to an mRNA transcribed from a nucleic acid molecule of claim 1 or a part thereof and can selectively bind to said mRNA or part thereof, said sequence being capable of inhibiting the synthesis of the protein encoded by said nucleic acid molecule.

11. A ribozyme characterized in that it is complementary to an mRNA transcribed from a nucleic acid molecule of claim 1 or a part thereof and can selectively bind to and cleave said mRNA or part thereof, thus inhibiting the synthesis of the protein encoded by said nucleic acid molecule.

12. An inhibitor characterized in that it can suppress the activity of the protein of claim 6.

13. A method for diagnosing a prostate carcinoma which comprises contacting a target sample suspected to contain the protein Trp8a, Trp8b, Trp10a and/or Trp10b or the Trp8a, Trp8b, Trp10a and/or Trp10b encoding mRNA with a reagent which reacts with Trp8a, Trp8b, Trp10a and/or Trp10b or the Trp8a, Trp8b, Trp10a and/or Trp10b encoding mRNA and detecting Trp8a, Trp8b, Trp10a and/or Trp10b or the Trp8a, Trp8b, Trp10a and/or Trp10b encoding mRNA.

14. The method of claim 13, wherein the reagent is a nucleic acid.

15. The method of claim 13, wherein the reagent is an antibody.

16. The method of claim 13, wherein the reagent is detectably labeled.

17. The method of claim 16, wherein the label is selected from the group consisting of a radioisotope, a bioluminescent compound, a chemiluminescent compound, a fluorescent compound, a metal chelate, or an enzyme.

18. A method for diagnosing an endometrial cancer (carcinoma of the uterus) which comprises contacting a target sample suspected to contain the protein Trp8a and/or Trp8b or the Trp8a and/or Trp8b encoding mRNA with a reagent which reacts with Trp8a and/or Trp8b or the Trp8a and/or Trp8a and/or trp8b encoding mRNA and detecting Trp8a and/or Trp8b or the Trp8a and/or Trp8b encoding mRNA.

19. The method of claim 18, wherein the reagent is a nucleic acid.

20. The method of claim 18, wherein the reagent is an antibody.

21. The method of claim 18, wherein the reagent is detectably labeled.

22. The method of claim 21, wherein the label is selected from the group consisting of a radioisotope, a bioluminescent compound, a chemiluminescent compound, a fluorescent compound, a metal chelate, or an enzyme.

23. A method for diagnosing a melanoma, chorion carcinoma, cancer of the lung and of the prostate in a tissue of a subject, comprising contacting a sample with a reagent which detects Trp10a and/or Trp10b antisense RNA or Trp10a and/or Trp10b related antisense RNA.

24. A method for preventing, treating, or ameliorating a prostate tumor, endometrial cancer (carcinoma of the uterus) tumor, a chorion carcinoma, cancer of the lung or melanoma, which comprises administering to a mammalian subject a therapeutically effective amount of a reagent which decreases or inhibits expression of Trp8a, Trp8b, Trp10a and/or Trp10b and/or the activity of Trp8a, Trp8b, Trp10a and/or Trp10b.

25. The method of claim 24, wherein the reagent is a nucleotide sequence comprising an antisense RNA.

26. The method of claim 24, wherein the reagent is a nucleotide sequence comprising a ribozyme.

27. The method of claim 24, wherein the reagent is an inhibitor of Trp8a, Trp8b, Trp10a and/or Trp10b.

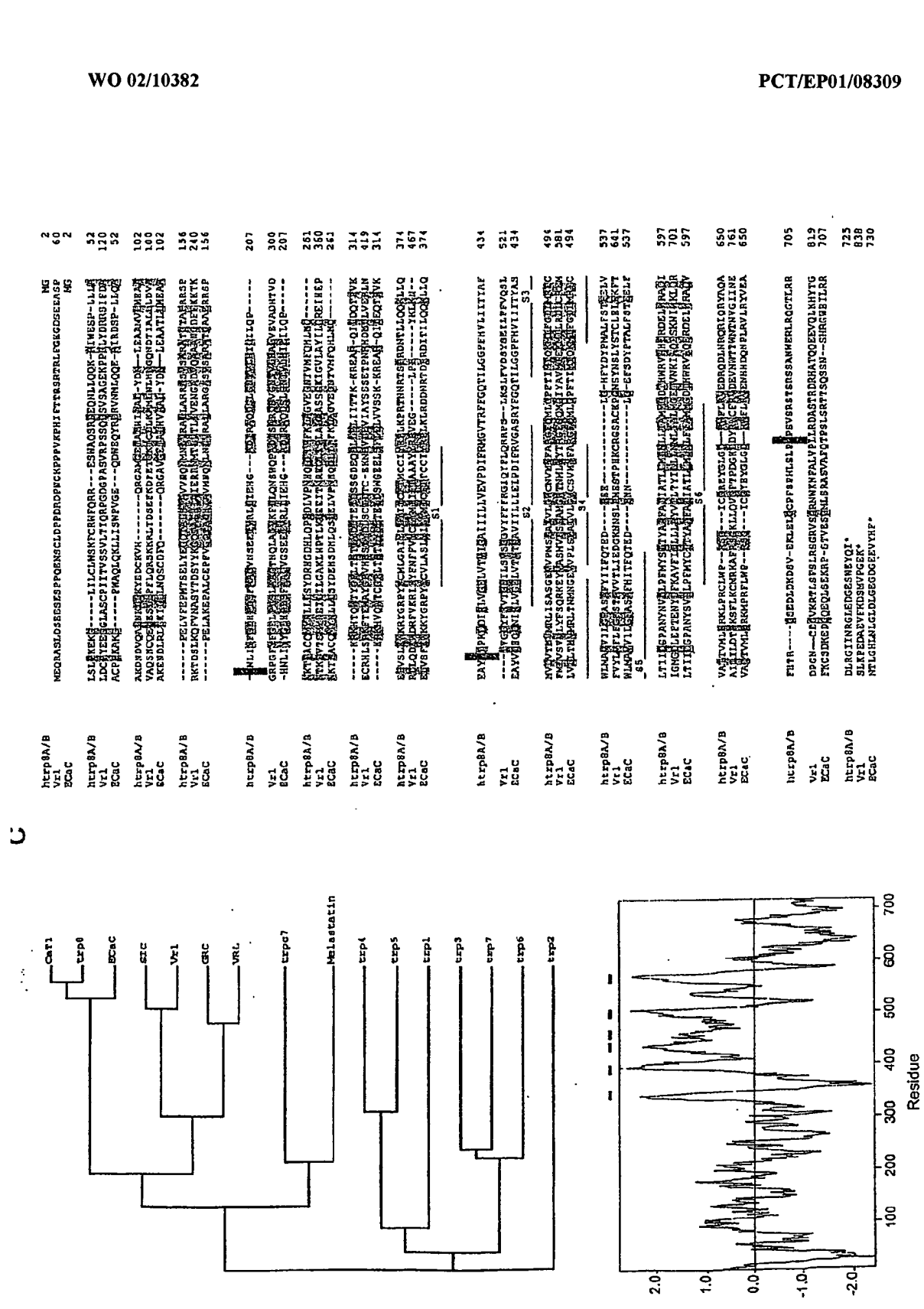
28. The method of claim 27, wherein the reagent is an anti-Trp8a-, anti Trp8b-, anti-Trp10a- and/or anti-Trp10b antibody or a fragment thereof.

29. A diagnostic kit useful for the detection of Trp8a, Trp8b, Trp10a and/or Trp10b or Trp8a, Trp8b, Trp10a and/or Trp10b encoding mRNA or Trp10a and/or Trp10b antisense transcripts in a sample, wherein the presence of an increased concentration of Trp8a, Trp8b, Trp9, Trp10a and/or Trp10b or Trp8a, Trp8b, Trp9, Trp10a and/or Trp10b encoding mRNA or Trp10a and/or Trp10b antisense transcripts is indicative for a prostate tumor, endometrial cancer (cancer of the uterus) tumor, a chorion carcinoma, cancer of the lung or melanoma, said kit comprising a probe for detection of Trp8a, Trp8b, Trp9, Trp10a or Trp10b or Trp8a, Trp8b, Trp9, Trp10a and/or Trp10b encoding mRNA or Trp10a and/or Trp10b antisense transcripts.

30. The kit of claim 29, wherein the target component to be detected is Trp8a, Trp8b, Trp9, Trp10a and/or Trp10b and the probe is an antibody.

31. A method for identifying a compound which acts as an agonist or antagonist on the ion channels Trp8, Trp9 and/or Trp10, said method comprising contacting a test compound with the ion channel Trp8, Trp9 and/or Trp10,and determining whether said test compound affects the calcium uptake.

Fig. 1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Cat1
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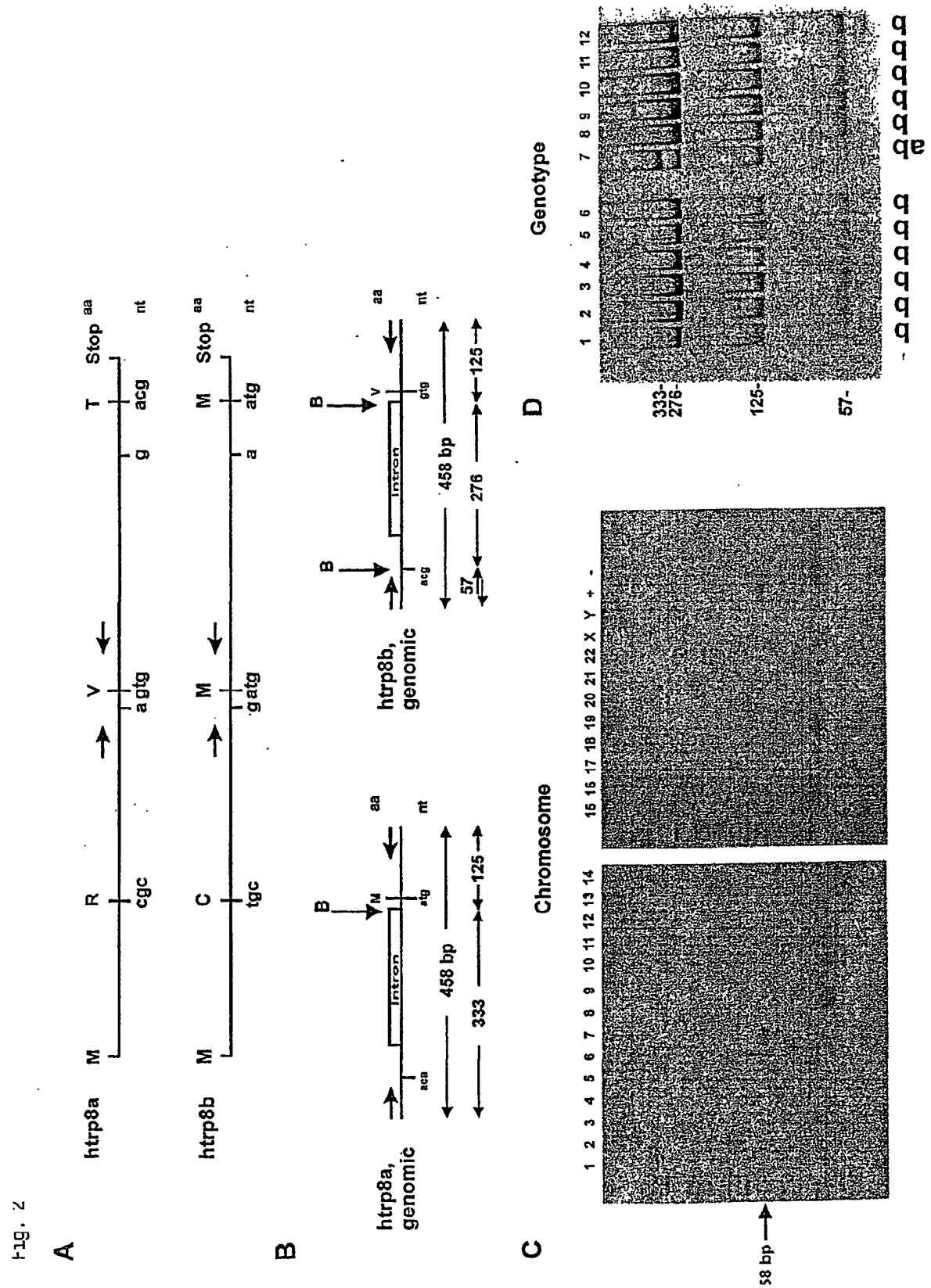
BCaC
GIC
Vrl
GNC
VRL
trp07
Melastatin
trp4
trp5
trp1
trp3
trp7
trp6
trp2
B
2.0
1.0
0.0
-1.0
-2.0
100
200
300
400
500
600
700
Residue
C
htrp8A/B
Vrl
BCaC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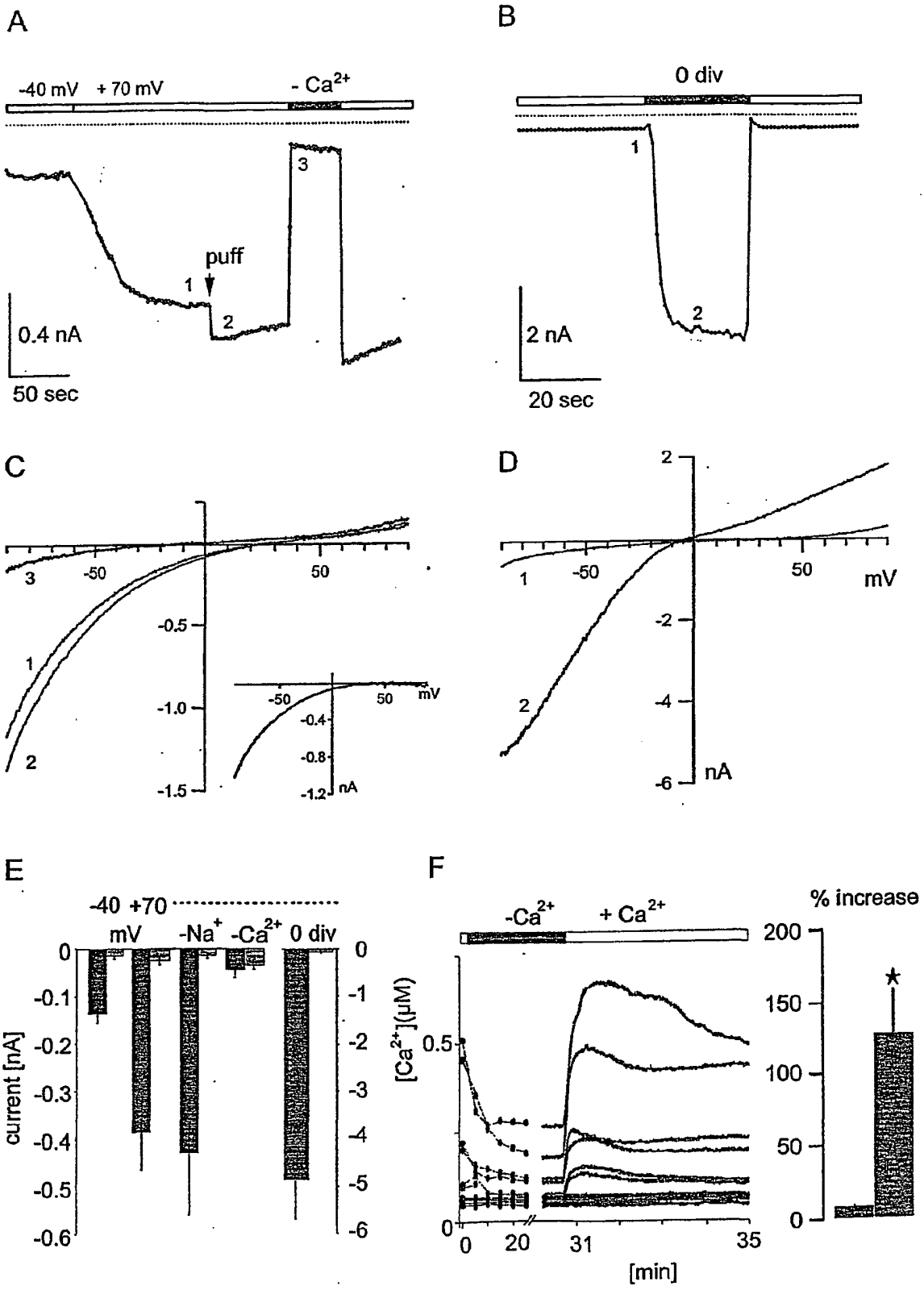
htrp8a
M
R
V
T
Stop
aa
nt
cgc
agtg
acg
htrp8b
M
C
M
M
Stop
aa
nt
tgc
atg
atg
B
htrp8a, genomic
htrp8b, genomic
Intron
458 bp
333
276
125
57
C
Chromosome
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 X Y + -
58 bp
D
Genotype
1 2 3 4 5 6 7 8 9 10 11 12
333-
276-
125-
57-
b
a

Fig. 2

Fig. 3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+ 70 mV
- Ca2+
puff
1
2
3
0.4 nA
50 sec
B
0 div
1
2
2 nA
20 sec
C
3
-50
50
1
2
-0.5
-1.0
-1.5
-50
50
mV
-0.4
-0.8
-1.2
nA
D
2
1
-50
50
mV
-2
-4
-6
nA
E
-40 +70
mV
-Na+
-Ca2+
0 div
current [nA]
0
-0.1
-0.2
-0.3
-0.4
-0.5
-0.6
-1
-2
-3
-4
-5
-6
F
-Ca2+
+ Ca2+
[Ca2+](µM)
0.5
0
0
20
31
35
[min]
% increase
200
150
100
50
0

Fig. 4

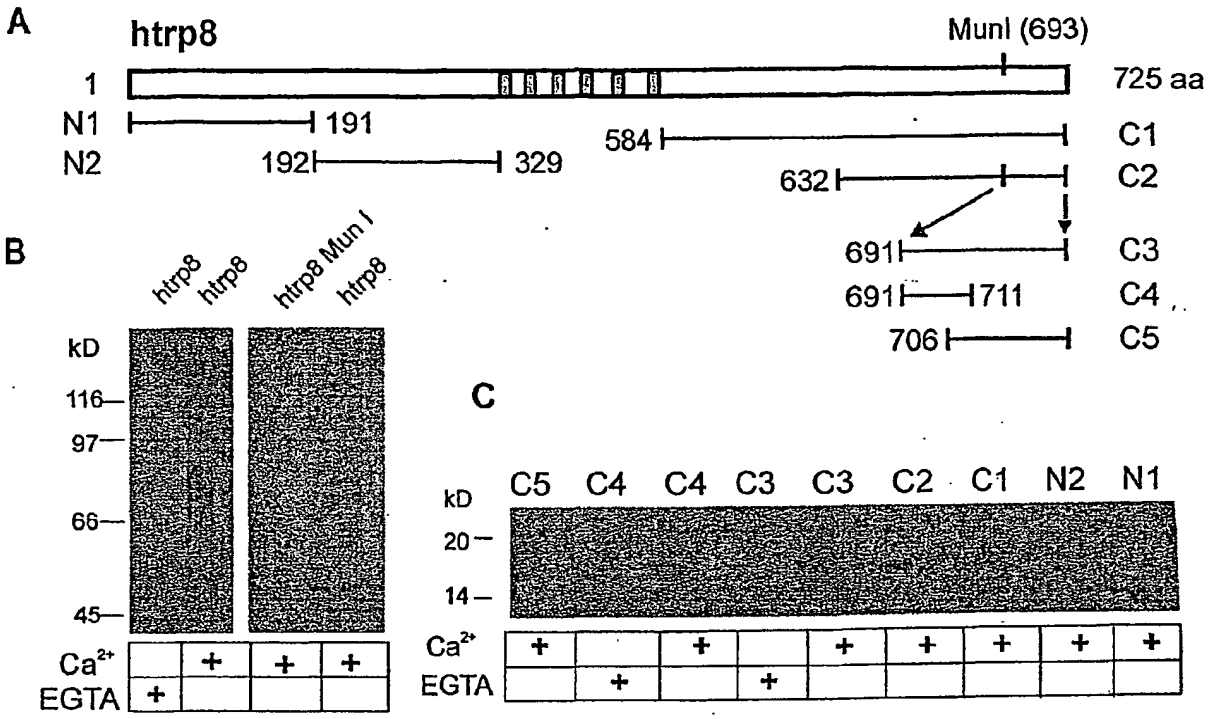
A
htrp8
MunI (693)
1
725 aa
N1
191
N2
192
329
584
C1
632
C2
691
C3
691
711
C4
706
C5
B
htrp8
htrp8
htrp8 Mun I
htrp8
kD
116
97
66
45
Ca2+
EGTA
C
kD
20
14
C5 C4 C4 C3 C3 C2 C1 N2 N1
Ca2+
EGTA

Fig. 5

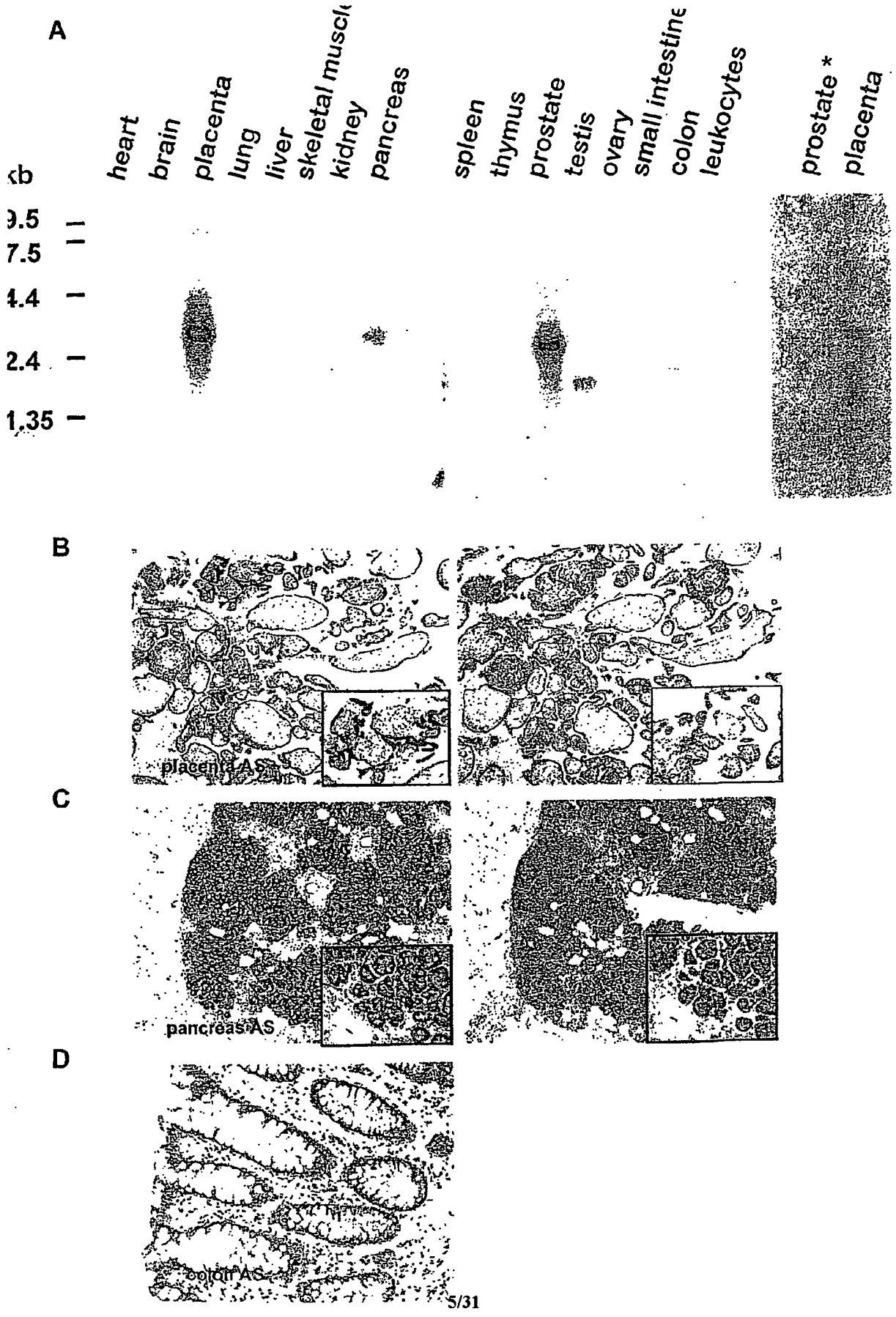
A
heart
brain
placenta
lung
liver
skeletal muscl
kidney
pancreas
spleen
thymus
prostate
testis
ovary
small intestine
colon
leukocytes
prostate *
placenta
kb
9.5
7.5
4.4
2.4
1.35
B
placenta AS
C
pancreas AS
D
colon AS

Fig. 6

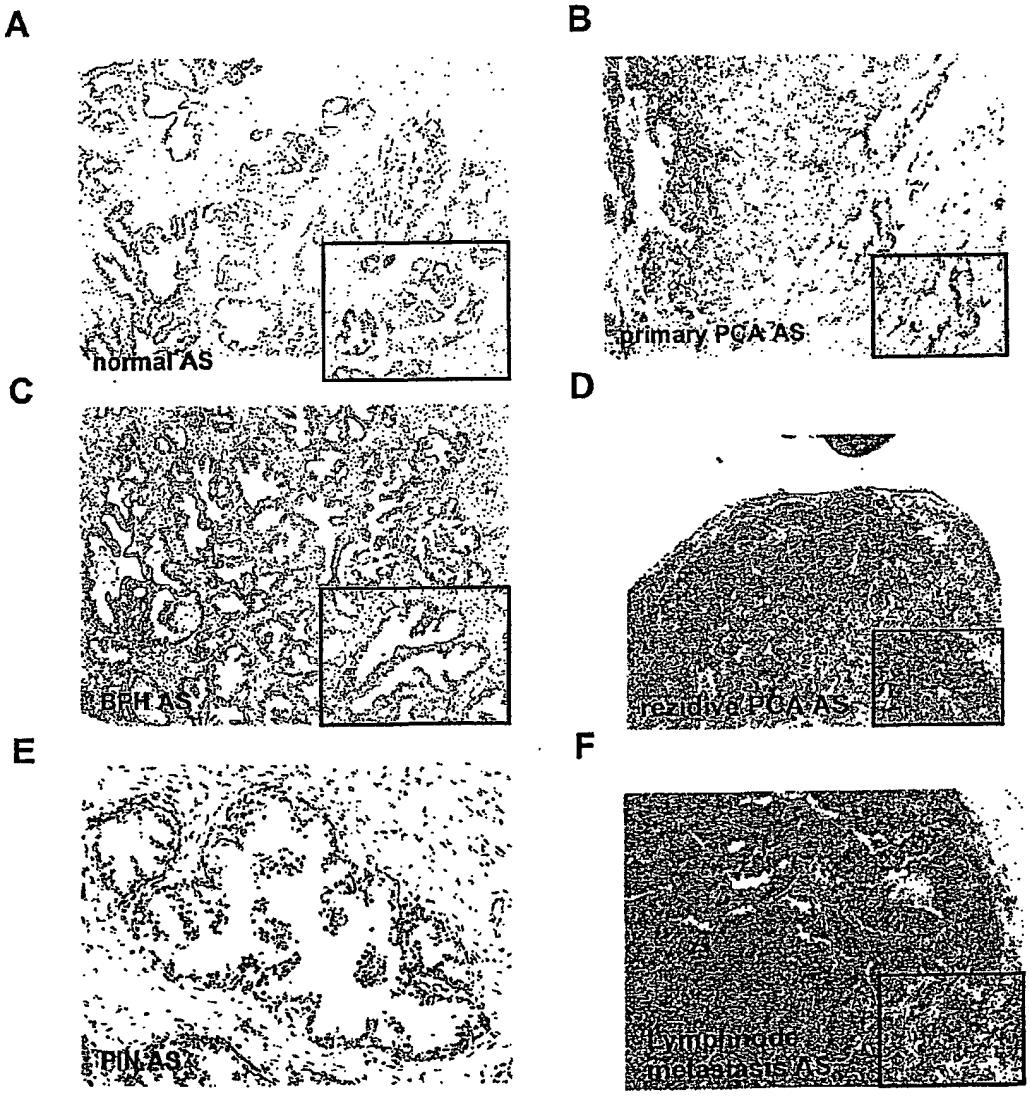
A
normal AS
B
primary PCA AS
C
BPH AS
D
rezidiva PCA AS
E
PIN AS
F
lymphnode
metastasis AS

Fig. 7

```
       10                  30                  50
GCCAAGTGTAACAAACTCACAGCCCTCTCCAAACTGGCTGGGGCTGCTGGGAGACTCCCA
       70                  90                 110
AGGAACTCGTCAGGAAGGCAGGAGACAGGAGACGGGACCTCTACAGGGAGACGGTGGGCC
      130                 150                 170
GGCCCTTGGGGGGGGCTGATGTGGCCCCAAGGCTGAGTCCCGTCAGGGTCTGGCCTCGGCC
      190                 210                 230
TCAGGCCCCCAAGGAGCCGGCCCTACACCCCATGGGTTTGTCACTGCCCAAGGAGAAAGG
                                M  G  L  S  L  P  K  E  K  G
      250                 270                 290
GCTAATTCTCTGCCTATGGAGCAAGTTCTGCAGATGGTTCCAGAGACGGGAGTCCTGGGC
 L  I  L  C  L  W  S  K  F  C  R  W  F  Q  R  R  E  S  W  A
      310                 330                 350
CCAGAGCCGAGATGAGCAGAACCTGCTGCAGCAGAAGAGGATCTGGGAGTCTCCTCTCCT
 Q  S  R  D  E  Q  N  L  L  Q  Q  K  R  I  W  E  S  P  L  L
      370                 390                 410
TCTAGCTGCCAAAGATAATGATGTCCAGGCCCTGAACAAGTTGCTCAAGTATGAGGATTG
 L  A  A  K  D  N  D  V  Q  A  L  N  K  L  L  K  Y  E  D  C
      430                 450                 470
CAAGGTGCACCAGAGAGGAGCCATGGGGGAAACAGCGCTACACATAGCAGCCCTCTATGA
 K  V  H  Q  R  G  A  M  G  E  T  A  L  H  I  A  A  L  Y  D
      490                 510                 530
CAACCTGGAGGCCGCCATGGTGCTGATGGAGGCTGCCCCGGAGCTGGTCTTTGAGCCCAT
 N  L  E  A  A  M  V  L  M  E  A  A  P  E  L  V  F  E  P  M
      550                 570                 590
GACATCTGAGCTCTATGAGGGTCAGACTGCACTGCACATCGCTGTTGTGAACCAGAACAT
 T  S  E  L  Y  E  G  Q  T  A  L  H  I  A  V  V  N  Q  N  M
      610                 630                 650
GAACCTGGTGCGAGCCCTGCTTGCCCGCAGGGCCAGTGTCTCTGCCAGAGCCACAGGCAC
 N  L  V  R  A  L  L  A  R  R  A  S  V  S  A  R  A  T  G  T
      670                 690                 710
TGCCTTCCGCCGTAGTCCCCGCAACCTCATCTACTTTGGGGAGCACCCTTTGTCCTTTGC
 A  F  R  R  S  P  R  N  L  I  Y  F  G  E  H  P  L  S  F  A
      730                 750                 770
TGCCTGTGTGAACAGTGAGGAGATCGTGCGGCTGCTCATTGAGCATGGAGCTGACATCCG
 A  C  V  N  S  E  E  I  V  R  L  L  I  E  H  G  A  D  I  R
      790                 810                 830
GGCCCAGGACTCCCTGGGAAACACAGTGTTACACATCCTCATCCTCCAGCCCAACAAAAC
 A  Q  D  S  L  G  N  T  V  L  H  I  L  I  L  Q  P  N  K  T
      850                 870                 890
CTTTGCCTGCCAGATGTACAACCTGTTGCTGTCCTACGACAGACATGGGGACCACCTGCA
 F  A  C  Q  M  Y  N  L  L  L  S  Y  D  R  H  G  D  H  L  Q
      910                 930                 950
GCCCCTGGACCTCGTGCCCAATCACCAGGGTCTCACCCCTTTCAAGCTGGCTGGAGTGGA
 P  L  D  L  V  P  N  H  Q  G  L  T  P  F  K  L  A  G  V  E
      970                 990                1010
GGGTAACACTGTGATGTTTCAGCACCTGATGCAGAAGCGGAAGCACACCCAGTGGACGTA
 G  N  T  V  M  F  Q  H  L  M  Q  K  R  K  H  T  Q  W  T  Y
     1030                1050                1070
TGGACCACTGACCTCGACTCTCTATGACCTCACAGAGATCGACTCCTCAGGGGATGAGCA
 G  P  L  T  S  T  L  Y  D  L  T  E  I  D  S  S  G  D  E  Q
     1090                1110                1130
GTCCCTGCTGGAACTTATCATCACCACCAAGAAGCGGGAGGCTCGCCAGATCCTGGACCA
 S  L  L  E  L  I  I  T  T  K  K  R  E  A  R  Q  I  L  D  Q
     1150                1170                1190
GACGCCGGTGAAGGAGCTGGTGAGCCTCAAGTGGAAGCGGTACGGGCGGCCGTACTTCTG
 T  P  V  K  E  L  V  S  L  K  W  K  R  Y  G  R  P  Y  F  C
     1210                1230                1250
CATGCTGGGTGCCATATATCTGCTGTACATCATCTGCTTCACCATGTGCTGCATCTACCG
 M  L  G  A  I  Y  L  L  Y  I  I  C  F  T  M  C  C  I  Y  R
     1270                1290                1310
```

Fig. 7 / continuation 1

```
CCCCCTCAAGCCCAGGACCAATAACCGCACAAGCCCCCGGGACAACACCCTCTTACAGCA
 P  L  K  P  R  T  N  N  R  T  S  P  R  D  N  T  L  L  Q  Q
      1330                1350                1370
GAAGCTACTTCAGGAAGCCTACGTGACCCCTAAGGACGATATCCGGCTGGTCGGGGAGCT
 K  L  L  Q  E  A  Y  V  T  P  K  D  D  I  R  L  V  G  E  L
      1390                1410                1430
GGTGACTGTCATTGGGGCTATCATCATCCTGCTGGTAGAGGTTCCAGACATCTTCAGAAT
 V  T  V  I  G  A  I  I  I  L  L  V  E  V  P  D  I  F  R  M
      1450                1470                1490
GGGGGTCACTCGCTTCTTTGGACAGACCATCCTTGGGGGCCCATTCCATGTCCTCATCAT
 G  V  T  R  F  F  G  Q  T  I  L  G  G  P  F  H  V  L  I  I
      1510                1530                1550
CACCTATGCCTTCATGGTGCTGGTGACCATGGTGATGCGGCTCATCAGTGCCAGCGGGGA
 T  Y  A  F  M  V  L  V  T  M  V  M  R  L  I  S  A  S  G  E
      1570                1590                1610
GGTGGTACCCATGTCCTTTGCACTCGTGCTGGGCTGGTGCAACGTCATGTACTTCGCCCG
 V  V  P  M  S  F  A  L  V  L  G  W  C  N  V  M  Y  F  A  R
      1630                1650                1670
AGGATTCCAGATGCTAGGCCCCTTCACCATCATGATTCAGAAGATGATTTTTGGCGACCT
 G  F  Q  M  L  G  P  F  T  I  M  I  Q  K  M  I  F  G  D  L
      1690                1710                1730
GATGCGATTCTGCTGGCTGATGGCTGTGGTCATCCTGGGCTTTGCTTCAGCCTTCTATAT
 M  R  F  C  W  L  M  A  V  V  I  L  G  F  A  S  A  F  Y  I
      1750                1770                1790
CATCTTCCAGACAGAGGACCCCGAGGAGCTAGGCCACTTCTACGACTACCCCATGGCCCT
 I  F  Q  T  E  D  P  E  E  L  G  H  F  Y  D  Y  P  M  A  L
      1810                1830                1850
GTTCAGCACCTTCGAGCTGTTCCTTACCATCATCGATGGCCCAGCCAACTACAACGTGGA
 F  S  T  F  E  L  F  L  T  I  I  D  G  P  A  N  Y  N  V  D
      1870                1890                1910
CCTGCCCTTCATGTACAGCATCACCTATGCTGCCTTTGCCATCATCGCCACACTGCTCAT
 L  P  F  M  Y  S  I  T  Y  A  A  F  A  I  I  A  T  L  L  M
      1930                1950                1970
GCTCAACCTCCTCATTGCCATGATGGGCGACACTCACTGGCGAGTGGCCCATGAGCGGGA
 L  N  L  L  I  A  M  M  G  D  T  H  W  R  V  A  H  E  R  D
      1990                2010                2030
TGAGCTGTGGAGGGCCCAGATTGTGGCCACCACGGTGATGCTGGAGCGGAAGCTGCCTCG
 E  L  W  R  A  Q  I  V  A  T  T  V  M  L  E  R  K  L  P  R
      2050                2070                2090
CTGCCTGTGGCCTCGCTCCGGGATCTGCGGACGGGAGTATGGCCTGGGGGACCGCTGGTT
 C  L  W  P  R  S  G  I  C  G  R  E  Y  G  L  G  D  R  W  F
      2110                2130                2150
CCTGCGGGTGGAAGACAGGCAAGATCTCAACCGGCAGCGGATCCAACGCTACGCACAGGC
 L  R  V  E  D  R  Q  D  L  N  R  Q  R  I  Q  R  Y  A  Q  A
      2170                2190                2210
CTTCCACACCCGGGGCTCTGAGGATTTGGACAAAGACTCAGTGGAAAAACTAGAGCTGGG
 F  H  T  R  G  S  E  D  L  D  K  D  S  V  E  K  L  E  L  G
      2230                2250                2270
CTGTCCCTTCAGCCCCCACCTGTCCCTTCCTACGCCCTCAGTGTCTCGAAGTACCTCCCG
 C  P  F  S  P  H  L  S  L  P  T  P  S  V  S  R  S  T  S  R
      2290                2310                2330
CAGCAGTGCCAATTGGGAAAGGCTTCGGCAAGGGACCCTGAGGAGAGACCTGCGTGGGAT
 S  S  A  N  W  E  R  L  R  Q  G  T  L  R  R  D  L  R  G  I
      2350                2370                2390
AATCAACAGGGGTCTGGAGGACGGGGAGAGCTGGGAATATCAGATCTGACTGCGTGTTCT
 I  N  R  G  L  E  D  G  E  S  W  E  Y  Q  I
      2410                2430                2450
CACTTCGCTTCCTGGAACTTGCTCTCATTTTCCTGGGTGCATCAAACAAAACAAAAACCA
      2470                2490                2510
AACACCCAGAGGTCTCATCTCCCAGGCCCCAGGGAGAAAGAGGAGTAGCATGAACGCCAA
      2530                2550                2570
GGAATGTACGTTGAGAATCACTGCTCCAGGCCTGCATTACTCCTTCAGCTCTGGGGCAGA
```

Fig. 7 / continuation 2

```
     2590                2610                2630
GGAAGCCCAGCCCAAGCACGGGGCTGGCAGGGCGTGAGGAACTCTCCTGTGGCCTGCTCA
     2650                2670                2690
TCACCCTTCCGACAGGAGCACTGCATGTCAGAGCACTTTAAAAACAGGCCAGCCTGCTTG
     2710                2730                2750
GGCCCTCGGTCTCCACCCCAGGGTCATAAGTGGGGAGAGAGCCCTTCCCAGGGCACCCAG
     2770                2790                2810
GCAGGTGCAGGGAAGTGCAGAGCTTGTGGAAAGCGTGTGAGTGAGGGAGACAGGAACGGC
     2830                2850                2870
TCTGGGGGTGGGAAGTGGGGCTAGGTCTTGCCAACTCCATCTTCAATAAAGTCGTTTTCG
     2890                2910
GATCCCTAAAAAAAAAAAAAAAAAAAAAAAAAA
```

```
MGLSLPKEKGLILCLWSKFCRWFQRRESWAQSRDEQNLLQQKRIWESPLLLAAKDNDVQALNKLLKYEDCKVHQRGAMGETALHIA
ALYDNLEAAMVLMEAAPELVFEPMTSELYEGQTALHIAVVNQNMNLVRALLARRASVSARATGTAFRRSPRNLIYFGEHPLSFAAC
VNSEEIVRLLIEHGADIRAQDSLGNTVLHILILQPNKTFACQMYNLLLSYDRHGDHLQPLDLVPNHQGLTPFKLAGVEGNTVMFQH
LMQKRKHTQWTYGPLTSTLYDLTEIDSSGDEQSLLELIITTKKREARQILDQTPVKELVSLKWKRYGRPYFCMLGAIYLLYIICFT
MCCIYRPLKPRTNNRTSPRDNTLLQQKLLQEAYVTPKDDIRLVGELVTVIGAIIILLVEVPDIFRMGVTRFFGQTILGGPFHVLII
TYAFMVLVTMVMRLISASGEVVPMSFALVLGWCNVMYFARGFQMLGPFTIMIQKMIFGDLMRFCWLMAVVILGFASAFYIIFQTED
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MLERKLPRCLWPRSGICGREYGLGDRWFLRVEDRQDLNRQRIQRYAQAFHTRGSEDLDKDSVEKLELGCPFSPHLSLPTPSVSRST
SRSSANWERLRQGTLRRDLRGIINRGLEDGESWEYQI
```

Figure 8:

A)

```
                                ATGGGTTTGTCACTGCCCAAGGAGAAAGGGCTAATTCTCT
                                M  G  L  S  L  P  K  E  K  G  L  I  L  C
        250                     270                     290
GCCTATGGAGCAAGTTCTGCAGATGGTTCCAGAGACGGGAGTCCTGGGCCCAGAGCCGAG
  L  W  S  K  F  C  R  W  F  Q  R  R  E  S  W  A  Q  S  R  D
        310                     330                     350
ATGAGCAGAACCTGCTGCAGCAGAAGAGGATCTGGGAGTCTCCTCTCCTTCTAGCTGCCA
  E  Q  N  L  L  Q  Q  K  R  I  W  E  S  P  L  L  L  A  A  K
        370                     390                     410
AAGATAATGATGTCCAGGCCCTGAACAAGTTGCTCAAGTATGAGGATTGCAAGGTGCACC
  D  N  D  V  Q  A  L  N  K  L  L  K  Y  E  D  C  K  V  H  Q
        430                     450                     470
AGAGAGGAGCCATGGGGGAAACAGCGCTACACATAGCAGCCCTCTATGACAACCTGGAGG
  R  G  A  M  G  E  T  A  L  H  I  A  A  L  Y  D  N  L  E  A
        490                     510                     530
CCGCCATGGTGCTGATGGAGGCTGCCCCGGAGCTGGTCTTTGAGCCCATGACATCTGAGC
  A  M  V  L  M  E  A  A  P  E  L  V  F  E  P  M  T  S  E  L
        550                     570                     590
TCTATGAGGGTCAGACTGCACTGCACATCGCTGTTGTGAACCAGAACATGAACCTGGTGC
  Y  E  G  Q  T  A  L  H  I  A  V  V  N  Q  N  M  N  L  V  R
        610                     630                     650
GAGCCCTGCTTGCCCGCAGGGCCAGTGTCTCTGCCAGAGCCACAGGCACTGCCTTCCGCC
  A  L  L  A  R  R  A  S  V  S  A  R  A  T  G  T  A  F  R  R
        670                     690                     710
GTAGTCCCTGCAACCTCATCTACTTTGGGGAGCACCCTTTGTCCTTTGCTGCCTGTGTGA
  S  P  C  N  L  I  Y  F  G  E  H  P  L  S  F  A  A  C  V  N
```

Fig. 8 / contin ı 1

```
      730                 750                 770
ACAGTGAGGAGATCGTGCGGCTGCTCATTGAGCATGGAGCTGACATCCGGGCCCAGGACT
  S  E  E  I  V  R  L  L  I  E  H  G  A  D  I  R  A  Q  D  S
      790                 810                 830
CCCTGGGAAACACAGTGTTACACATCCTCATCCTCCAGCCCAACAAAACCTTTGCCTGCC
  L  G  N  T  V  L  H  I  L  I  L  Q  P  N  K  T  F  A  C  Q
      850                 870                 890
AGATGTACAACCTGTTGCTGTCCTACGACAGACATGGGGACCACCTGCAGCCCCTGGACC
  M  Y  N  L  L  L  S  Y  D  R  H  G  D  H  L  Q  P  L  D  L
      910                 930                 950
TCGTGCCCAATCACCAGGGTCTCACCCCTTTCAAGCTGGCTGGAGTGGAGGGTAACACTG
  V  P  N  H  Q  G  L  T  P  F  K  L  A  G  V  E  G  N  T  V
      970                 990                1010
TGATGTTTCAGCACCTGATGCAGAAGCGGAAGCACACCCAGTGGACGTATGGACCACTGA
  M  F  Q  H  L  M  Q  K  R  K  H  T  Q  W  T  Y  G  P  L  T
     1030                1050                1070
CCTCGACTCTCTATGACCTCACAGAGATCGACTCCTCAGGGGATGAGCAGTCCCTGCTGG
  S  T  L  Y  D  L  T  E  I  D  S  S  G  D  E  Q  S  L  L  E
     1090                1110                1130
AACTTATCATCACCACCAAGAAGCGGGAGGCTCGCCAGATCCTGGACCAGACGCCGGTGA
  L  I  I  T  T  K  K  R  E  A  R  Q  I  L  D  Q  T  P  V  K
     1150                1170                1190
AGGAGCTGGTGAGCCTCAAGTGGAAGCGGTACGGGCGGCCGTACTTCTGCATGCTGGGTG
  E  L  V  S  L  K  W  K  R  Y  G  R  P  Y  F  C  M  L  G  A
     1210                1230                1250
CCATATATCTGCTGTACATCATCTGCTTCACCATGTGCTGCATCTACCGCCCCCTCAAGC
  I  Y  L  L  Y  I  I  C  F  T  M  C  C  I  Y  R  P  L  K  P
     1270                1290                1310
CCAGGACCAATAACCGCACGAGCCCCCGGGACAACACCCTCTTACAGCAGAAGCTACTTC
  R  T  N  N  R  T  S  P  R  D  N  T  L  L  Q  Q  K  L  L  Q
     1330                1350                1370
AGGAAGCCTACATGACCCCTAAGGACGATATCCGGCTGGTCGGGGAGCTGGTGACTGTCA
  E  A  Y  M  T  P  K  D  D  I  R  L  V  G  E  L  V  T  V  I
     1390                1410                1430
TTGGGGCTATCATCATCCTGCTGGTAGAGGTTCCAGACATCTTCAGAATGGGGGTCACTC
  G  A  I  I  I  L  L  V  E  V  P  D  I  F  R  M  G  V  T  R
     1450                1470                1490
GCTTCTTTGGACAGACCATCCTTGGGGGCCCATTCCATGTCCTCATCATCACCTATGCCT
  F  F  G  Q  T  I  L  G  G  P  F  H  V  L  I  I  T  Y  A  F
     1510                1530                1550
TCATGGTGCTGGTGACCATGGTGATGCGGCTCATCAGTGCCAGCGGGGAGGTGGTACCCA
  M  V  L  V  T  M  V  M  R  L  I  S  A  S  G  E  V  V  P  M
     1570                1590                1610
TGTCCTTTGCACTCGTGCTGGGCTGGTGCAACGTCATGTACTTCGCCCGAGGATTCCAGA
  S  F  A  L  V  L  G  W  C  N  V  M  Y  F  A  R  G  F  Q  M
     1630                1650                1670
TGCTAGGCCCCTTCACCATCATGATTCAGAAGATGATTTTTGGCGACCTGATGCGATTCT
  L  G  P  F  T  I  M  I  Q  K  M  I  F  G  D  L  M  R  F  C
     1690                1710                1730
GCTGGCTGATGGCTGTGGTCATCCTGGGCTTTGCTTCAGCCTTCTATATCATCTTCCAGA
  W  L  M  A  V  V  I  L  G  F  A  S  A  F  Y  I  I  F  Q  T
     1750                1770                1790
CAGAGGACCCCGAGGAGCTAGGCCACTTCTACGACTACCCCATGGCCCTGTTCAGCACCT
  E  D  P  E  E  L  G  H  F  Y  D  Y  P  M  A  L  F  S  T  F
     1810                1830                1850
TCGAGCTGTTCCTTACCATCATCGATGGCCCAGCCAACTACAACGTGGACCTGCCCTTCA
  E  L  F  L  T  I  I  D  G  P  A  N  Y  N  V  D  L  P  F  M
     1870                1890                1910
TGTACAGCATCACCTATGCTGCCTTTGCCATCATCGCCACACTGCTCATGCTCAACCTCC
  Y  S  I  T  Y  A  A  F  A  I  I  A  T  L  L  M  L  N  L  L
     1930                1950                1970
TCATTGCCATGATGGGCGACACTCACTGGCGAGTGGCCCATGAGCGGGATGAGCTGTGGA
```

Fig. 8 / continuation 2

```
  I  A  M  M  G  D  T  H  W  R  V  A  H  E  R  D  E  L  W  R
     1990              2010              2030
GGGCCCAGATTGTGGCCACCACGGTGATGCTGGAGCGGAAGCTGCCTCGCTGCCTGTGGC
  A  Q  I  V  A  T  T  V  M  L  E  R  K  L  P  R  C  L  W  P
     2050              2070              2090
CTCGCTCCGGGATCTGCGGACGGGAGTATGGCCTGGGAGACCGCTGGTTCCTGCGGGTGG
  R  S  G  I  C  G  R  E  Y  G  L  G  D  R  W  F  L  R  V  E
     2110              2130              2150
AAGACAGGCAAGATCTCAACCGGCAGCGGATCCAACGCTACGCACAGGCCTTCCACACCC
  D  R  Q  D  L  N  R  Q  R  I  Q  R  Y  A  Q  A  F  H  T  R
     2170              2190              2210
GGGGCTCTGAGGATTTGGACAAAGACTCAGTGGAAAAACTAGAGCTGGGCTGTCCCTTCA
  G  S  E  D  L  D  K  D  S  V  E  K  L  E  L  G  C  P  F  S
     2230              2250              2270
GCCCCCACCTGTCCCTTCCTATGCCCTCAGTGTCTCGAAGTACCTCCCGCAGCAGTGCCA
  P  H  L  S  L  P  M  P  S  V  S  R  S  T  S  R  S  S  A  N
     2290              2310              2330
ATTGGGAAAGGCTTCGGCAAGGGACCCTGAGGAGAGACCTGCGTGGGATAATCAACAGGG
  W  E  R  L  R  Q  G  T  L  R  R  D  L  R  G  I  I  N  R  G
     2350              2370              2390
GTCTGGAGGACGGGGAGAGCTGGGAATATCAGATCTGA
  L  E  D  G  E  S  W  E  Y  Q  I  *
```

```
MGLSLPKEKGLILCLWSKFCRWFQRRESWAQSRDEQNLLQQKRIWESPLLLAAKDNDVQALNKLLKYEDCKVHQRGAMGETALHIA
ALYDNLEAAMVLMEAAPELVFEPMTSELYEGQTALHIAVVNQNMNLVRALLARRASVSARATGTAFRRSPCNLIYFGEHPLSFAAC
VNSEEIVRLLIEHGADIRAQDSLGNTVLHILILQPNKTFACQMYNLLLSYDRHGDHLQPLDLVPNHQGLTPFKLAGVEGNTVMFQH
LMQKRKHTQWTYGPLTSTLYDLTEIDSSGDEQSLLELIITTKKREARQILDQTPVKELVSLKWKRYGRPYFCMLGAIYLLYIICFT
MCCIYRPLKPRTNNRTSPRDNTLLQQKLLQEAYMTPKDDIRLVGELVTVIGAIIILLVEVPDIFRMGVTRFFGQTILGGPFHVLII
TYAFMVLVTMVMRLISASGEVVPMSFALVLGWCNVMYFARGFQMLGPFTIMIQKMIFGDLMRFCWLMAVVILGFASAFYIIFQTED
PEELGHFYDYPMALFSTFELFLTIIDGPANYNVDLPFMYSITYAAFAIIATLLMLNLLIAMMGDTHWRVAHERDELWRAQIVATTV
MLERKLPRCLWPRSGICGREYGLGDRWFLRVEDRQDLNRQRIQRYAQAFHTRGSEDLDKDSVEKLELGCPFSPHLSLPMPSVSRST
SRSSANWERLRQGTLRRDLRGIINRGLEDGESWEYQI
```

B)

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CAAACTCACAGCCCTCTCCAAACTGGCTGGGGCTGCTGGGAGACTCCCAAGGAACTCGTCAGGAAGGCAGGAGACAGGAGACGGGA
CCTCTACAGGGAGACGGTGGGCCGGCCCTTGGGGGGGCTGATGTGGCCCCAAGGCTGAGTCCCGTCAGGGTCTGGCCTCGGCCTCA
GGCCCCCAAGGAGCCGGCCCTACACCCCATGGGTTTGTCACTGCCCAAGGAGAAAGGGCTAATTCTCTGCCTATGGAGCAAGTTCT
GCAGATGGTTCCAGAGACGGGAGTCCTGGGCCCAGAGCCGAGATGAGCAGAACCTGCTGCAGCAGAAGAGGATCTGGGAGTCTCCT
CTCCTTCTAGCTGCCAAAGATAATGATGTCCAGGCCCTGAACAAGTTGCTCAAGTATGAGGATTGCAAGGTGCACCAGAGAGGAGC
CATGGGGGAAACAGCGCTACACATAGCAGCCCTCTATGACAACCTGGAGGCCGCCATGGTGCTGATGGAGGCTGCCCCGGAGCTGG
TCTTTGAGCCCATGACATCTGAGCTCTATGAGGGTCAGACTGCACTGCACATCGCTGTTGTGAACCAGAACATGAACCTGGTGCGA
GCCCTGCTTGCCCGCAGGGCCAGTGTCTCTGCCAGAGCCACAGGCACTGCCTTCCGCCGTAGTCCCCGCAACCTCATCTACTTTGG
GGAGCACCCTTTGTCCTTTGCTGCCTGTGTGAACAGTGAGGAGATCGTGCGGCTGCTCATTGAGCATGGAGCTGACATCCGGGCCC
AGGACTGCCTGGCCCAACAAAACCTTTGCCTGCCAGATGTACAACCTGTTGCTGTCCTACGACAGACATGGGGACCACCTGCAGCC
CCTGGACCTCGTGCCCAATCACCAGGGTCTCACCCCTTTCAAGCTGGCTGGAGTGGAGGGTAACACTGTGATGTTTCAGCACCTGA
TGCAGAAGCGGAAGCACACCCAGTGGACGTATGGACCACTGACCTCGACTCTCTATGACCTCACAGAGATCGACTCCTCAGGGGAT
GAGCAGTCCCTGCTGGAACTTATCATCACCACCAAGAAGCGGGAGGCTCGCCAGATCCTGGACCAGACGCCGGTGAAGGAGCTGGT
GAGCCTCAAGTGGAAGCGGTACGGGCGGCCGTACTTCTGCATGCTGGGTGCCATATATCTGCTGTACATCATCTGCTTCACCATGT
GCTGCATCTACCGCCCCCTCAAGCCCAGGACCAATAACCGCACAAGCCCCCGGGACAACACCCTCTTACAGCAGAAGCTACTTCAG
GAAGCCTACGTGACCCCTAAGGACGATATCCGGCTGGTCGGGGAGCTGGTGACTGTCATTGGGGCTATCATCATCCTGCTGGTAGA
GGTTCCAGACATCTTCAGAATGGGGGTCACTCGCTTCTTTGGACAGACCATCCTTGGGGGCCCATTCCATGTCCTCATCATCACCT
ATGCCTTCATGGTGCTGGTGACCATGGTGATGCGGCTCATATGATTTTTGGCGACCTGATGCGATTCTGCTGGCTGATGGCTGTGG
TCATCCTGGGCTTTGCTTCAGCCTTCTATATCATCTTCCAGACAGAGGACCCCGAGGAGCTAGGCCACTTCTACGACTACCCCATG
GCCCTGTTCAGCACCTTCGAGCTGGTCCTTACCATCATCGATGGCCCAGCCAACTACAACGTGGACCTGCCCTTCATGTACAGCAT
CACCTATGCTGCCTTTGCCATCATCGCCACACTGCTCATGCTCAACCTCCTCATTGCCATGATGGGCGACACTCACTGGCGAGTGG
CCCATGAGCGGGATGAGCTGTGGAGGGCCCAGATTGTGGCCACCACGGTGATGCTGGAGCGGAAGCTGCCTCGCTGCCTGTGGCCT
CGCTCCGGGATCTGCGGACGGGAGTATGGCCTGGGGGACCGCTGGTTCCTGCGGGTGGAAGACAGGCAAGATCTCAACCGGCAGCG
```

Fig. 8 / continuation 3

GATCCAACGCTACGCACAGGCCTTCCACACCCGGGGCTCTGAGGATTTGGACAAAGACTCAGTGGAAAAACTAGAGCTGGGCTGTC
CCTTCAGCCCCCACCTGTCCCTTCCTACGCCCTCAGTGTCTCGAAGTACCTCCCGCAGCAGTGCCAATTGGGAAAGGCTTCGGCAA
GGGACCCTGAGGAGAGACCTGCGTGGGATAATCAACAGGGGTCTGGAGGACGGGGAGAGCTGGGAATATCAGATCTGACTGCGTGT
TCTCACTTCGCTTCCTGGAACTTGCTCTCATTTTCCTGGGTGCATCAAACAAAACAAAAACCAAACACCCAGAGGTCTCATCTCCC
AGGCCCCAGGGAGAAAGAGGAGTAGCATGAACGCCAAGGAATGTACGTTGAGAATCACTGCTCCAGGCCTGCATTACTCCTTCAGC
TCTGGGGCAGAGGAAGCCCAGCCCAAGCACGGGGCTGGCAGGGCGTGAGGAACTCTCCTGTGGCCTGCTCATCACCCTTCCGACAG
GAGCACTGCATGTCAGAGCACTTTAAAAACAGGCCAGCCTGCTTGGGCCCTCGGTCTCCACCCCAGGGTCATAAGTGGGGAGAGAG
CCCTTCCCAGGGCACCCAGGCAGGTGCAGGGAAGTGCAGAGCTTGTGGAAAGCGTGTGAGTGAGGGAGACAGGAACGGCTCTGGGG
GTGGGAAGTGGGGCTAGGTCTTGCCAACTCCATCTTCAATAAAGTCGTTTTCGGATCCCTAAAAAAAAAAAAAAAAAAAAAAAAAA

c.)

CAAACTCACAGCCCTCTCCAAACTGGCTGGGGCTGCTGGGAGACTCCCAAGGAACTCGTCAGGAAGGCAGGAGACAGGAGACGGGA
CCTCTACAGGGAGACGGTGGGCCGGCCCTTGGGGGGGCTGATGTGGCCCCAAGGCTGAGTCCCGTCAGGGTCTGGCCTCGGCCTCA
GGCCCCCAAGGAGCCGGCCCTACACCCCATGGGTTTGTCACTGCCCAAGGAGAAAGGGCTAATTCTCTGCCTATGGAGCAAGTTCT
GCAGATGGTTCCAGAGACGGGAGTCCTGGGCCCAGAGCCGAGATGAGCAGAACCTGCTGCAGCAGAAGAGGATCTGGGAGTCTCCT
CTCCTTCTAGCTGCCAAAGATAATGATGTCCAGGCCCTGAACAAGTTGCTCAAGTATGAGGATTGCAAGGTGCACCAGAGAGGAGC
CATGGGGGAAACAGCGCTACACATAGCAGCCCTCTATGACAACCTGGAGGCCGCCATGGTGCTGATGGAGGCTGCCCCGGAGCTGG
TCTTTGAGCCCATGACATCTGAGCTCTATGAGGTCCTGACTGCCCATCACTTGAACGCCTGCCCCCTGAAATGCCAGGGCCTAGAG
AAGAGGAAGAGATGGGCAGCAGCTGGATCCCCTGGGAATCCTGAACACCCGAGAGCTCCCTGTTCTCCATCCCAGGCTACCCCTGA
GGGAAAGAGACTGGGGTGCATATGGGAGGGACCCCCTGCAGGATCCTGGGGACAGACCCGTGACTGACAGCTGTCTCTGGGCCAGG
TCAGACTGCACTGCACATCGCTGTTGTGAACCAGAACATGAACCTGGTGCGAGCCCTGCTTGCCCGCAGGGCCAGTGTCTCTGCCA
GAGCCACAGGCACTGCCTTCCGCCGTAGTCCCTGCAACCTCATCTACTTTGGGGAGCACCCTTTGTCCTTTGCTGCCTGTGTGAAC
AGTGAGGAGATCGTGCGGCTGCTCATTGAGCATGGAGCTGACATCCGGGCCCAGGACTCCCTGGCCCAACAAAACCTTTGCCTGCC
AGATGTACAACCTGTTGCTGTCCTACGACAGACATGGGGACCACCTGCAGCCCCTGGACCTCGTGCCCAATCACCAGGGTCTCACC
CCTTTCAAGCTGGCTGGAGTGGAGGGTAACACTGTGATGTTTCAGCACCTGATGCAGAAGCGGAAGCACACCCAGTGGACGTATGG
ACCACTGACCTCGACTCTCTATGACCTCACAGAGATCGACTCCTCAGGGGATGAGCAGTCCCTGCTGGAACTTATCATCACCACCA
AGAAGCGGGAGGCTCGCCAGATCCTGGACCAGACGCCGGTGAAGGAGCTGGTGAGCCTCAAGTGGAAGCGGTACGGGCGGCCGTAC
TTCTGCATGCTGGGTGCCATATATCTGCTGTACATCATCTGCTTCACCATGTGCTGCATCTACCGCCCCCTCAAGCCCAGGACCAA
TAACCGCACGAGCCCCCGGGACAACACCCTCTTACAGCAGAAGCTACTTCAGGAAGCCTACATGACCCCTAAGGACGATATCCGGC
TGGTCGGGGAGCTGGTGACTGTCATTGGGGCTATCATCATCCTGCTGGTAGAGGTTCCAGACATCTTCAGAATGGGGGTCACTCGC
TTCTTTGGACAGACCATCCTTGGGGGCCCATTCCATGTCCTCATCATCACCTATGCCTTCATGGTGCTGGTGACCATGGTGATGCG
GCTCATCAGTGCCAGCGGGGAGGTGGTACCCATGTCCTTTGCACTCGTGCTGGGCTGGTGCAACGTCATGTACTTCGCCCGAGGAT
TCCAGATGCTAGGCCCCTTCACCATCATGATTCAGAAGATGATTTTTGGCGACCTGATGCGATTCTGCTGGCTGATGGCTGTGGTC
ATCCTGGGCTTTGCTTAGACAGAGGACCCCGAGGAGCTAGGCCACTTCTACGACTACCCCATGGCCCTGTTCAGCACCTTCGAGCT
GGTCCTTACCATCATCGATGGCCCAGCCAACTACAACGTGGACCTGCCCTTCATGTACAGCATCACCTATGCTGCCTTTGCCATCA
TCGCCACACTGCTCATGCTCAACCTCCTCATTGCCATGATGGGCGACACTCACTGGCGAGTGGCCCATGAGCGGGATGAGCTGTGG
AGGGCCCAGATTGTGGCCACCACGGTGATGCTGGAGCGGAAGCTGCCTCGCTGCCTGTGGCCTCGCTCCGGGATCTGCGGACGGGA
GTATGGCCTGGGAGACCGCTGGTTCCTGCGGGTGGAAGACAGGCAAGATCTCAACCGGCAGCGGATCCAACGCTACGCACAGGCCT
TCCACACCCGGGGCTCTGAGGATTTGGACAAAGACTCAGTGGAAAAACTAGAGCTGGGCTGTCCCTTCAGCCCCCACCTGTCCCTT
CCTATGCCCTCAGTGTCTCGAAGTACCTCCCGCAGCAGTGCCAATTGGGAAAGGCTTCGGCAAGGGACCCTGAGGAGAGACCTGCG
TGGGATAATCAACAGGGGTCTGGAGGACGGGGAGAGCTGGGAATATCAGATCTGACTGCGTGTTCTCACTTCGCTTCCTGGAACTT
GCTCTCATTTTCCTGGGTGCATCAAACAAAACAAAAACCAAACACCCAGAGGTCTCATCTCCCAGGCCCCAGGGAGAAAGAGGAGT
AGCATGAACGCCAAGGAATGTACGTTGAGAATCACTGCTCCAGGCCTGCATTACTCCTTCAGCTCTGGGGCAGAGGAAGCCCAGCC
CAAGCACGGGGCTGGCAGGGCGTGAGGAACTCTCCTGTGGCCTGCTCATCACCCTTCCGACAGGAGCACTGCATGTCAGAGCACTT
TAAAAACAGGCCAGCCTGCTTGGGCCCTCGGTCTCCACCCAGGGTCATAAGTGGGGAGAGAGCCCTTCCCAGGGCACCCAGGCAG
GTGCAGGGAAGTGCAGAGCTTGTGGAAAGCGTGTGAGTGAGGGAGACAGGAACGGCTCTGGGGGTGGGAAGTGGGGCTAGGTCTTG
CCAACTCCATCTTCAATAAAGTCGTTTTCGGATCCCTAAAAAAAAAAAAAAAAAAAAAAAAAA

D.)

CAAACTCACAGCCCTCTCCAAACTGGCTGGGGCTGCTGGGAGACTCCCAAGGAACTCGTCAGGAAGGCAGGAGACAGGAGACGGGA
CCTCTACAGGGAGACGGTGGGCCGGCCCTTGGGGGGGCTGATGTGGCCCCAAGGCTGAGTCCCGTCAGGGTCTGGCCTCGGCCTCA
GGCCCCCAAGGAGCCGGCCCTACACCCCATGGGTTTGTCACTGCCCAAGGAGAAAGGGCTAATTCTCTGCCTATGGAGCAAGTTCT
GCAGATGGTTCCAGAGACGGGAGTCCTGGGCCCAGAGCCGAGATGAGCAGAACCTGCTGCAGCAGAAGAGGATCTGGGAGTCTCCT
CTCCTTCTAGCTGCCAAAGATAATGATGTCCAGGCCCTGAACAAGTTGCTCAAGTATGAGGATTGCAAGGTGCACCAGAGAGGAGC
CATGGGGGAAACAGCGCTACACATAGCAGCCCTCTATGACAACCTGGAGGCCGCCATGGTGCTGATGGAGGCTGCCCCGGAGCTGG
TCTTTGAGCCCATGACATCTGAGCTCTATGAGGGTCAGACTGCACTGCACATCGCTGTTGTGAACCAGAACATGAACCTGGTGCGA
GCCCTGCTTGCCCGCAGGGCCAGTGTCTCTGCCAGAGCCACAGGCACTGCCTTCCGCCGTAGTCCCCGCAACCTCATCTACTTTGG

Fig. 8 / continuation

AAACACAGTGTTACACATCCTCATCCTCCAGCCCAACAAAACCTTTGCCTGCCAGATGTACAACCTGTTGCTGTCCTACGACAGAC
ATGGGGACCACCTGCAGCCCCCTGGACCTCGTGCCCAATCACCAGGGTCTCACCCCTTTCAAGCTGGCTGGAGTGGAGGGTAACACT
GTGATGTTTCAGCACCTGATGCAGAAGCGGAAGCACACCCAGTGGACGTATGGACCACTGACCTCGACTCTCTATGACCTCACAGA
GATCGACTCCTCAGGGGATGAGCAGTCCCTGCTGGAACTTATCATCACCACCAAGAAGCGGGAGGCTCGCCAGATCCTGGACCAGA
CGCCGGTGAAGGAGCTGGTGAGCCTCAAGTGGAAGCGGTACGGGCGGCCGTACTTCTGCATGCTGGGTGCCATATATCTGCTGTAC
ATCATCTGCTTCACCATGTGCTGCATCTACCGCCCCCTCAAGCCCAGGACCAATAACCGCACAAGCCCCCGGGACAACACCCTCTT
ACAGCAGAAGCTACTTCAGGAAGCCTACGTGACCCCTAAGGACGATATCCGGCTGGTCGGGGAGCTGGTGACTGTCATTGGGGCTA
TCATCATCCTGCTGGTAGAGGTTCCAGACATCTTCAGAATGGGGGTCACTCGCTTCTTTGGACAGACCATCCTTGGGGGCCCATTC
CATGTCCTCATCATCACCTATGCCTTCATGGTGCTGGTGACCATGGTGATGCGGCTCATCAGTGCCAGCGGGGAGGTGGTACCCAT
GTCCTTTGCACTCGTGCTGGGCTGGTGCAACGTCATGTACTTCGCCCGAGGATTCCAGATGCTAGGCCCCTTCACCATCATGATTC
AGAAGATGATTTTTGGCGACCTGATGCGATTCTGCTGGCTGATGGCTGTGGTCATCCTGGGCTTTGCTTCAGCCTTCTATATCATC
TTCCAGACAGAGGACCCCGAGGAGCTAGGCCACTTCTACGACTACCCCATGGCCCTGTTCAGCACCTTCGAGCTGGTCCTTACCAT
CATCGATGGCCCAGCCAACTACAACGTGGACCTGCCCTTCATGTACAGCATCACCTATGCTGCCTTTGCCATCATCGCCACACTGC
TCATGCTCAACCTCCTCATTGCCATGATGGGCGACACTCACTGGCGAGTGGCCCATGAGCGGGATGAGCTGTGGAGGGCCCAGATT
GTGGCCACCACGGTGATGCTGGAGCGGAAGCTGCCTCGCTGCCTGTGGCCTCGCTCCGGGATCTGCGGACGGGAGTATGGCCTGGG
GGACCGCTGGTTCCTGCGGGTGGAAGACAGGCAAGATCTCAACCGGCAGCGGATCCAACGCTACGCACAGGCCTTCCACACCCGGG
GCTCTGAGGATTTGGACAAAGACTCAGTGGAAAAACTAGAGCTGGGCTGTCCCTTCAGCCCCCACCTGTCCCTTCCTACGCCCTCA
GTGTCTCGAAGTACCTCCCGCAGCAGTGCCAATTGGGAAAGGCTTCGGCAAGGGACCCTGAGGAGAGACCTGCGTGGGATAATCAA
CAGGGGTCTGGAGGACGGGGAGAGCTGGGAATATCAGATCTGACTGCGTGTTCTCACTTCGCTTCCTGGAACTTGCTCTCATTTTC
CTGGGTGCATCAAACAAAACAAAAACCAAACACCCAGAGGTCTCATCTCCCAGGCCCCAGGGAGAAAGAGGAGTAGCATGAACGCC
AAGGAATGTACGTTGAGAATCACTGCTCCAGGCCTGCATTACTCCTTCAGCTCTGGGGCAGAGGAAGCCCAGCCCAAGCACGGGGC
TGGCAGGGCGTGAGGAACTCTCCTGTGGCCTGCTCATCACCCTTCCGACAGGAGCACTGCATGTCAGAGCACTTTAAAAACAGGCC
AGCCTGCTTGGGCCCTCGGTCTCCACCCCAGGGTCATAAGTGGGGAGAGAGCCCTTCCCAGGGCACCCAGGCAGGTGCAGGGAAGT
GCAGAGCTTGTGGAAAGCGTGTGAGTGAGGGAGACAGGAACGGCTCTGGGGGTGGGAAGTGGGGCTAGGTCTTGCCAACTCCATCT
TCAATAAAGTCGTTTTCGGATCCCTAAAAAAAAAAAAAAAAAAAAAAAAA

E.)

CACACATGGGGCCTCCCAGGAGTGCCCAGGACCTCGTGCTGTTGGCCTCTGAATCTATCGTCTCCAATCCGCTGTCCCACAGAAGC
CATATAACCCACCTCTCTGTAAATGCCAGGAGCCATGGGGGAAACAGCGCTACACATAGCAGCCCTCTATGACAACCTGGAGGCCG
CCATGGTGCTGATGGAGGCTGCCCCGGAGCTGGTCTTTGAGCCCATGACATCTGAGCTCTATGGAGGGTGAGGGCCCACGGGTCTG
GGGTGAAGAGCAGGAGTGACGTGGTTGGGTATTCAAGTCAGTCTCTGTGATGGATAATTTGGGAAAGACACAGGGGATCTGAGCCT
CCTACTCTTTTTTSTCTTCTCTGTCTCCCTTCCGTGTCAGTCCCTGACTGCCCATCACTTGAACGCCTGCCCCCTGAAATGCCAGGG
GCCTAGAGAAGAGGAAGAGATGGGCAGCAGCTGGATCCCCTGGGAATCCTGAACACCCGAGAGCTCCCTGTTCTCCATCCCAGGCT
ACCCCTGAGGGAAAGAGACTAGGGGTGCATATGGGAGGGACCCCCTGCAGGATCCTAGGGGACAGACCCGTGACTGACAGCTGTCT
CTGGGCCAGGTCAGACTGCACTGCACATCGCTGTTGTGAACCAGAACATGAACCTGGTGCGAGCCCTGCTTGCCCGCAGGGCCAGT
GTCTCTGCCAGAGCCACAGGCACTGCCTTCCGCCGTAGTCCCTGCAACCTCATCTACTTTGGGGAGCACCCTTTGTCCTTTGCTGC
CTGTGTGAACAGTGAGGAGATCGTGCGGCTGCTCATTGAGCATGGAGCTGACATCCGGGCCCAGGACTCCCTGGATGTACAACCTG
TTGCTGTCCTACGACAGACATGGGGACCACCTGCAGCCCCTGGACCTCGTGCCCAATCACCAGGGTCTCACCCCTTTCAAGCTGGC
TGGAGTGGAGGGTAACACTGTGATGTTTCAGCACCTGATGCAGAAGCGGAAGCACACCCAGTGGACGTATGGACCACTGACCTCGA
CTCTCTATGACCTCACAGAGATCGACTCCTCAGGGGATGAGCAGTCCCTGCTGGAACTTATCATCACCACCAAGAAGCGGGAGGCT
CGCCAGATCCTGGACCAGACGCCGGTGAAGGAGCTGGTGAGCCTCAAGTGGAAGCGGTACGGGCGGCCGTACTTCTGCATGCTGGG
TGCCATATATCTGCTGTACATCATCTGCTTCACCATGTGCTGCATCTACCGCCCCCTCAAGCCCAGGACCAATAACCGCACGAGCC
CCCGGGACAACACCCTCTTACAGCAGAAGCTACTTCAGGAAGCCTACATGACCCCTAAGGACGATATCCGGCTGGTCGGGGAGCTG
GTGACTGTCATTGGGGCTATCATCATCCTGCTGGTAGAGGTTCCAGACATCTTCAGAATGGGGGTCACTCGCTTCTTTGGACAGAC
CATCCTTGGGGGCCCATTCCATGTCCTCATCATCACCTATGCCTTCATGGTGCTGGTGACCATGGTGATGCGGCTCATCAGTGCCA
GCGGGGAGGTGGTACCCATGTCCTTTGCACTCGTGCTGGGCTGGTGCAACGTCATGTACTTCGCCCGAGGATTCCAGATGCTAGGC
CCCTTCACCATCATGATTCAGAAGATGATTTTTGGCGACCTGATGCGATTCTGCTGGCTGATGGCTGTGGTCATCCTGGGCTTTGC
TTCAGCCTTCTATATCATCTTCCAGACAGAGGACCCCGAGGAGCTAGGCCACTTCTACGACTACCCCATGGCCCTGTTCAGCACCT
TCGAGCTGGTCCTTACCATCATCGATGGCCCAGCCAACTACAACGTGGACCTGCCCTTCATGTACAGCATCACCTATGCTGCCTTT
GCCATCATCGCCACACTGCTCATGCTCAACCTCCTCATTGCCATGATGGGCGACACTCACTGGCGAGTGGCCCATGAGCGGGATGA
GCTGTGGAGGGCCCAGATTGTGGCCACCACGGTGATGCTGGAGCGGAAGCTGCCTCGCTGCCTGTGGCCTCGCTCCGGGATCTGCG
GACGGGAGTATGGCCTGGGAGACCGCTGGTTCCTGCGGGTGGAAGACAGGCAAGATCTCAACCGGCAGCGGATCCAACGCTACGCA
CAGGCCTTCCACACCCGGGGCTCTGAGGATTTGGACAAAGACTCAGTGGAAAAACTAGAGCTGGGCTGTCCCTTCAGCCCCCACCT
GTCCCTTCCTATGCCCTCAGTGTCTCGAAGTACCTCCCGCAGCAGTGCCAATTGGGAAAGGCTTCGGCAAGGGACCCTGAGGAGAG
ACCTGCGTGGGATAATCAACAGGGGTCTGGAGGACGGGGAGAGCTGGGAATATCAGATCTGACTGCGTGTTCTCACTTCGCTTCCT
GGAACTTGCTCTCATTTTCCTGGGTGCATCAAACAAAACAAAAACCAAACACCCAGAGGTCTCATCTCCCAGGCCCCAGGGAGAAA
GAGGAGTAGCATGAACGCCAAGGAATGTACGTTGAGAATCACTGCTCCAGGCCTGCATTACTCCTTCAGCTCTGGGGCAGAGGAAG
CCCAGCCCAAGCACGGGGCTGGCAGGGCGTGAGGAACTCTCCTGTGGCCTGCTCATCACCCTTCCGACAGGAGCACTGCATGTCAG
AGCACTTTAAAAACAGGCCAGCCTGCTTGGGCCCTCGGTCTCCACCCCAGGGTCATAAGTGGGGAGAGAGCCCTTCCCAGGGCACC

Fig. 8 / continuation 5

CAGGCAGGTGCAGGGAAGTGCAGAGCTTGTGGAAAGCGTGTGAGTGAGGGAGACAGGAACGGCTCTGGGGGTGGGAAGTGGGGCTA
GGTCTTGCCAACTCCATCTTCAATAAAGTCGTTTTCGGATCCCTAAAAAAAAAAAAAAAAAAAAAAAAAAA

Figure 9:

A.

```
        10                  30                  50
CGGGGCCCTGGGCTGCAGGAGGTTGCGGCGGCCGCGGCAGCATGGTGGTGCCGGAGAAGG
                                            M  V  V  P  E  K  E
        70                  90                 110
AGCAGAGCTGGATCCCCAAGATCTTCAAGAAGAAGACCTGCACGACGTTCATAGTTGACT
  Q  S  W  I  P  K  I  F  K  K  K  T  C  T  T  F  I  V  D  S
       130                 150                 170
CCACAGATCCGGGAGGGACCTTGTGCCAGTGTGGGCGCCCCCGGACCGCCCACCCCGCAG
  T  D  P  G  G  T  L  C  Q  C  G  R  P  R  T  A  H  P  A  V
       190                 210                 230
TGGCCATGGAGGATGCCTTCGGGGCAGCCGTGGTGACCGTGTGGGACAGCGATGCACACA
  A  M  E  D  A  F  G  A  A  V  V  T  V  W  D  S  D  A  H  T
       250                 270                 290
CCACGGAGAAGCCCACCGATGCCTACGGAGAGCTGGACTTCACGGGGGCCGGCCGCAAGC
  T  E  K  P  T  D  A  Y  G  E  L  D  F  T  G  A  G  R  K  H
       310                 330                 350
ACAGCAATTTCCTCCGGCTCTCTGACCGAACGGATCCAGCTGCAGTTTATAGTCTGGTCA
  S  N  F  L  R  L  S  D  R  T  D  P  A  A  V  Y  S  L  V  T
       370                 390                 410
CACGCACATGGGGCTTCCGTGCCCCGAACCTGGTGGTGTCAGTGCTGGGGGGATCGGGGG
  R  T  W  G  F  R  A  P  N  L  V  V  S  V  L  G  G  S  G  G
       430                 450                 470
GCCCCGTCCTCCAGACCTGGCTGCAGGACCTGCTGCGTCGTGGGCTGGTGCGGGCTGCCC
  P  V  L  Q  T  W  L  Q  D  L  L  R  R  G  L  V  R  A  A  Q
       490                 510                 530
AGAGCACAGGAGCCTGGATTGTCACTGGGGGTCTGCACACGGGCATCGGCCGGCATGTTG
  S  T  G  A  W  I  V  T  G  G  L  H  T  G  I  G  R  H  V  G
       550                 570                 590
GTGTGGCTGTACGGGACCATCAGATGGCCAGCACTGGGGGCACCAAGGTGGTGGCCATGG
  V  A  V  R  D  H  Q  M  A  S  T  G  G  T  K  V  V  A  M  G
       610                 630                 650
GTGTGGCCCCCTGGGGTGTGGTCCGGAATAGAGACACCCTCATCAACCCCAAGGGCTCGT
  V  A  P  W  G  V  V  R  N  R  D  T  L  I  N  P  K  G  S  F
       670                 690                 710
TCCCTGCGAGGTACCGGTGGCGCGGTGACCCGGAGGACGGGGTCCAGTTTCCCCTGGACT
  P  A  R  Y  R  W  R  G  D  P  E  D  G  V  Q  F  P  L  D  Y
       730                 750                 770
ACAACTACTCGGCCTTCTTCCTGGTGGACGACGGCACACACGGCTGCCTGGGGGGCGAGA
  N  Y  S  A  F  F  L  V  D  D  G  T  H  G  C  L  G  G  E  N
       790                 810                 830
ACCGCTTCCGCTTGCGCCTGGAGTCCTACATCTCACAGCAGAAGACGGGCGTGGGAGGGA
  R  F  R  L  R  L  E  S  Y  I  S  Q  Q  K  T  G  V  G  G  T
       850                 870                 890
CTGGAATTGACATCCCTGTCCTGCTCCTCCTGATTGATGGTGATGAGAAGATGTTGACGC
  G  I  D  I  P  V  L  L  L  L  I  D  G  D  E  K  M  L  T  R
       910                 930                 950
GAATAGAGAACGCCACCCAGGCTCAGCTCCCATGTCTCCTCGTGGCTGGCTCAGGGGGAG
  I  E  N  A  T  Q  A  Q  L  P  C  L  L  V  A  G  S  G  G  A
       970                 990                1010
CTGCGGACTGCCTGGCGGAGACCCTGGAAGACACTCTGGCCCCAGGGAGTGGGGGAGCCA
  A  D  C  L  A  E  T  L  E  D  T  L  A  P  G  S  G  G  A  R
      1030                1050                1070
GGCAAGGCGAAGCCCGAGATCGAATCAGGCGTTTCTTTCCCAAAGGGGACCTTGAGGTCC
```

Fig. 9 / continuation 1

```
  Q  G  E  A  R  D  R  I  R  R  F  F  P  K  G  D  L  E  V  L
     1090              1110              1130
TGCAGGCCCAGGTGGAGAGGATTATGACCCGGAAGGAGCTCCTGACAGTCTATTCTTCTG
  Q  A  Q  V  E  R  I  M  T  R  K  E  L  L  T  V  Y  S  S  E
     1150              1170              1190
AGGATGGGTCTGAGGAATTCGAGACCATAGTTTTGAAGGCCCTTGTGAAGGCCTGTGGGA
  D  G  S  E  E  F  E  T  I  V  L  K  A  L  V  K  A  C  G  S
     1210              1230              1250
GCTCGGAGGCCTCAGCCTACCTGGATGAGCTGCGTTTGGCTGTGGCTTGGAACCGCGTGG
  S  E  A  S  A  Y  L  D  E  L  R  L  A  V  A  W  N  R  V  D
     1270              1290              1310
ACATTGCCCAGAGTGAACTCTTTCGGGGGGACATCCAATGGCGGTCCTTCCATCTCGAAG
  I  A  Q  S  E  L  F  R  G  D  I  Q  W  R  S  F  H  L  E  A
     1330              1350              1370
CTTCCCTCATGGACGCCCTGCTGAATGACCGGCCTGAGTTCGTGCGCTTGCTCATTTCCC
  S  L  M  D  A  L  L  N  D  R  P  E  F  V  R  L  L  I  S  H
     1390              1410              1430
ACGGCCTCAGCCTGGGCCACTTCCTGACCCCGATGCGCCTGGCCCAACTCTACAGCGCGG
  G  L  S  L  G  H  F  L  T  P  N  R  L  A  Q  L  Y  S  A  A
     1450              1470              1490
CGCCCTCCAACTCGCTCATCCGCAACCTTTTGGACCAGGCGTCCCACAGCGCAGGCACCA
  P  S  N  S  L  I  R  N  L  L  D  Q  A  S  H  S  A  G  T  K
     1510              1530              1550
AAGCCCCAGCCCTAAAAGGGGGAGCTGCGGAGCTCCGGCCCCCTGACGTGGGGCATGTGC
  A  P  A  L  K  G  G  A  A  E  L  R  P  P  D  V  G  H  V  L
     1570              1590              1610
TGAGGATGCTGCTGGGGAAGATGTGCGCGCCGAGGTACCCCTCCGGGGGCGCCTGGGACC
  R  M  L  L  G  K  M  C  A  P  R  Y  P  S  G  G  A  W  D  P
     1630              1650              1670
CTCACCCAGGCCAGGGCTTCGGGGAGAGCATGTATCTGCTCTCGGACAAGGCCACCTCGC
  H  P  G  Q  G  F  G  E  S  M  Y  L  L  S  D  K  A  T  S  P
     1690              1710              1730
CGCTCTCGCTGGATGCTGGCCTCGGGCAGGCCCCCTGGAGCGACCTGCTTCTTTGGGCAC
  L  S  L  D  A  G  L  G  Q  A  P  W  S  D  L  L  L  W  A  L
     1750              1770              1790
TGTTGCTGAACAGGGCACAGATGGCCATGTACTTCTGGGAGATGGGTTCCAATGCAGTTT
  L  L  N  R  A  Q  M  A  M  Y  F  W  E  M  G  S  N  A  V  S
     1810              1830              1850
CCTCAGCTCTTGGGGCCTGTTTGCTGCTCCGGGTGATGGCACGCCTGGAGCCTGACGCTG
  S  A  L  G  A  C  L  L  L  R  V  M  A  R  L  E  P  D  A  E
     1870              1890              1910
AGGAGGCAGCACGGAGGAAAGACCTGGCGTTCAAGTTTGAGGGGATGGGCGTTGACCTCT
  E  A  A  R  R  K  D  L  A  F  K  F  E  G  M  G  V  D  L  F
     1930              1950              1970
TTGGCGAGTGCTATCGCAGCAGTGAGGTGAGGGCTGCCCGCCTCCTCCTCCGTCGCTGCC
  G  E  C  Y  R  S  S  E  V  R  A  A  R  L  L  L  R  R  C  P
     1990              2010              2030
CGCTCTGGGGGGATGCCACTTGCCTCCAGCTGGCCATGCAAGCTGACGCCCGTGCCTTCT
  L  W  G  D  A  T  C  L  Q  L  A  M  Q  A  D  A  R  A  F  F
     2050              2070              2090
TTGCCCAGGATGGGGTACAGTCTCTGCTGACACAGAAGTGGTGGGGAGATATGGCCAGCA
  A  Q  D  G  V  Q  S  L  L  T  Q  K  W  W  G  D  M  A  S  T
     2110              2130              2150
CTACACCCATCTGGGCCCTGGTTCTCGCCTTCTTTTGCCCTCCACTCATCTACACCCGCC
  T  P  I  W  A  L  V  L  A  F  F  C  P  P  L  I  Y  T  R  L
     2170              2190              2210
TCATCACCTTCAGGAAATCAGAAGAGGAGCCCACACGGGAGGAGCTAGAGTTTGACATGG
  I  T  F  R  K  S  E  E  E  P  T  R  E  E  L  E  F  D  M  D
     2230              2250              2270
ATAGTGTCATTAATGGGGAAGGGCCTGTCGGGACGGCGGACCCAGCCGAGAAGACGCCGC
  S  V  I  N  G  E  G  P  V  G  T  A  D  P  A  E  K  T  P  L
     2290              2310              2330
```

Fig. 9 / continuation 2

```
TGGGGGTCCCGCGCCAGTCGGGCCGTCCGGGTTGCTGCGGGGGCCGCTGCGGGGGGCGCC
  G  V  P  R  Q  S  G  R  P  G  C  C  G  G  R  C  G  G  R  R
     2350                2370                2390
GGTGCCTACGCCGCTGGTTCCACTTCTGGGGCGTGCCGGTGACCATCTTCATGGGCAACG
  C  L  R  R  W  F  H  F  W  G  V  P  V  T  I  F  M  G  N  V
     2410                2430                2450
TGGTCAGCTACCTGCTGTTCCTGCTGCTTTTCTCGCGGGTGCTGCTCGTGGATTTCCAGC
  V  S  Y  L  L  F  L  L  L  F  S  R  V  L  L  V  D  F  Q  P
     2470                2490                2510
CGGCGCCGCCCGGCTCCCTGGAGCTGCTGCTCTATTTCTGGGCTTTCACGCTGCTGTGCG
  A  P  P  G  S  L  E  L  L  L  Y  F  W  A  F  T  L  L  C  E
     2530                2550                2570
AGGAACTGCGCCAGGGCCTGAGCGGAGGCGGGGGCAGCCTCGCCAGCGGGGGCCCCGGGC
  E  L  R  Q  G  L  S  G  G  G  G  S  L  A  S  G  G  P  G  P
     2590                2610                2630
CTGGCCATGCCTCACTGAGCCAGCGCCTGCGCCTCTACCTCGCCGACAGCTGGAACCAGT
  G  H  A  S  L  S  Q  R  L  R  L  Y  L  A  D  S  W  N  Q  C
     2650                2670                2690
GCGACCTAGTGGCTCTCACCTGCTTCCTCCTGGGCGTGGGCTGCCGGCTGACCCCGGGTT
  D  L  V  A  L  T  C  F  L  L  G  V  G  C  R  L  T  P  G  L
     2710                2730                2750
TGTACCACCTGGGCCGCACTGTCCTCTGCATCGACTTCATGGTTTTCACGGTGCGGCTGC
  Y  H  L  G  R  T  V  L  C  I  D  F  M  V  F  T  V  R  L  L
     2770                2790                2810
TTCACATCTTCACGGTCAACAAACAGCTGGGGCCCAAGATCGTCATCGTGAGCAAGATGA
  H  I  F  T  V  N  K  Q  L  G  P  K  I  V  I  V  S  K  M  M
     2830                2850                2870
TGAAGGACGTGTTCTTCTTCCTCTTCTTCCTCGGCGTGTGGCTGGTAGCCTATGGCGTGG
  K  D  V  F  F  F  L  F  F  L  G  V  W  L  V  A  Y  G  V  A
     2890                2910                2930
CCACGGAGGGGCTCCTGAGGCCACGGGACAGTGACTTCCCAAGTATCCTGCGCCGCGTCT
  T  E  G  L  L  R  P  R  D  S  D  F  P  S  I  L  R  R  V  F
     2950                2970                2990
TCTACCGTCCCTACCTGCAGATCTTCGGGCAGATTCCCCAGGAGGACATGGACGTGGCCC
  Y  R  P  Y  L  Q  I  F  G  Q  I  P  Q  E  D  M  D  V  A  L
     3010                3030                3050
TCATGGAGCACAGCAACTGCTCGTCGGAGCCCGGCTTCTGGGCACACCCTCCTGGGGCCC
  M  E  H  S  N  C  S  S  E  P  G  F  W  A  H  P  P  G  A  Q
     3070                3090                3110
AGGCGGGCACCTGCGTCTCCCAGTATGCCAACTGGCTGGTGGTGCTGCTCCTCGTCATCT
  A  G  T  C  V  S  Q  Y  A  N  W  L  V  V  L  L  L  V  I  F
     3130                3150                3170
TCCTGCTCGTGGCCAACATCCTGCTGGTCAACTTGCTCATTGCCATGTTCAGTTACACAT
  L  L  V  A  N  I  L  L  V  N  L  L  I  A  M  F  S  Y  T  F
     3190                3210                3230
TCGGCAAAGTACAGGGCAACAGCGATCTCTACTGGAAGGCGCAGCGTTACCGCCTCATCC
  G  K  V  Q  G  N  S  D  L  Y  W  K  A  Q  R  Y  R  L  I  R
     3250                3270                3290
GGGAATTCCACTCTCGGCCCGCGCTGGCCCCGCCCTTTATCGTCATCTCCCACTTGCGCC
  E  F  H  S  R  P  A  L  A  P  P  F  I  V  I  S  H  L  R  L
     3310                3330                3350
TCCTGCTCAGGCAATTGTGCAGGCGACCCCGGAGCCCCCAGCCGTCCTCCCCGGCCCTCG
  L  L  R  Q  L  C  R  R  P  R  S  P  Q  P  S  S  P  A  L  E
     3370                3390                3410
AGCATTTCCGGGTTTACCTTTCTAAGGAAGCCGAGCGGAAGCTGCTAACGTGGGAATCGG
  H  F  R  V  Y  L  S  K  E  A  E  R  K  L  L  T  W  E  S  V
     3430                3450                3470
TGCATAAGGAGAACTTTCTGCTGGCACGCGCTAGGGACAAGCGGGAGAGCGACTCCGAGC
  H  K  E  N  F  L  L  A  R  A  R  D  K  R  E  S  D  S  E  R
     3490                3510                3530
GTCTGAAGCGCACGTCCCAGAAGGTGGACTTGGCACTGAAACAGCTGGGACACATCCGCG
  L  K  R  T  S  Q  K  V  D  L  A  L  K  Q  L  G  H  I  R  E
```

Fig. 9 / continue 3

```
     3550                3570                3590
AGTACGAACAGCGCCTGAAAGTGCTGGAGCGGGAGGTCCAGCAGTGTAGCCGCGTCCTGG
  Y  E  Q  R  L  K  V  L  E  R  E  V  Q  Q  C  S  R  V  L  G
     3610                3630                3650
GGTGGGTGGCCGAGGCCCTGAGCCGCTCTGCCTTGCTGCCCCCAGGTGGGCCGCCACCCC
  W  V  A  E  A  L  S  R  S  A  L  L  P  P  G  G  P  P  P  P
     3670                3690                3710
CTGACCTGCCTGGGTCCAAAGACTGAGCCCTGCTGGCGGACTTCAAGGAGAAGCCCCCAC
  D  L  P  G  S  K  D  *
     3730                3750                3770
AGGGGATTTTGCTCCTAGAGTAAGGCTCATCTGGGCCTCGGCCCCCGCACCTGGTGGCCT
     3790                3810                3830
TGTCCTTGAGGTGAGCCCCATGTCCATCTGGGCCACTGTCAGGACCACCTTTGGGAGTGT
     3850                3870                3890
CATCCTTACAAACCACAGCATGCCCGGCTCCTCCCAGAACCAGTCCCAGCCTGGGAGGAT
     3910                3930                3950
CAAGGCCTGGATCCCGGGCCGTTATCCATCTGGAGGCTGCAGGGTCCTTGGGGTAACAGG
     3970                3990                4010
GACCACAGACCCCTCACCACTCACAGATTCCTCACACTGGGGAAATAAAGCCATTTCAGA
     4030
GGAAAAAAAAAAAAAAAAAAAA
```

```
MVVPEKEQSWIPKIFKKKTCTTFIVDSTDPGGTLCQCGRPRTAHPAVAMEDAFGAAVVTVWDSDAHTTEKPTDAYELDFTGAG
SNFLRLSDRTDPAAVYSLVTRTWGFRAPNLVVSVLGGSGGPVLQTWLQDLLRRGLVRAAQSTGAWIVTGGLHTGIGRHVGVAV
QMASTGGTKVVAMGVAPWGVVRNRDTLINPKGSFPARYRWRGDPEDGVQFPLDYNYSAFFLVDDGTHGCLGGENRFRLRLESY
QKTGVGGTGIDIPVLLLLIDGDEKNLTRIENATQAHVPCLLVAGSRGLGMPGGTLEAHLAQDGDHKANQSTNQLLLPKDLSLQ
SIDRKTLQSYSERLAVAWNRVDIAQSELFRGDIQWRSFHLEASLMDALLNDRPEFVRLLISHGLSLGHFLTPMRLAQLYSAAF
LIRNLLDQASHSAGTKAPALKGGAAELRPPDVGHVLRMLLGKMCAPRYPSGGAWDPHPGQGFGESMYLLSDKATSPLSLDAGI
PWSDLLLWALLLNRAQMAMYFWEMGSNAVSSALGACLLLRVMARLEPDAEEAARRKDLAFKFEGMGVDLFGECYRSSEVRAAF
RRCPLNGDATCLQLAMQADARAFFAQDGVQSLLTQKWWGDMASTTPIWALVLAFFCPPLIYTRLITFRKSBEEPTREELEFDM
INGEGPVGTADPAEKTPLGVPRQSGRPGCCGGRCGGRRCLRRWFHFWGVPVTIFMGNVVSYLLFLLLFSRVLLVDFQPAPPGS
LLYFWAFTLLCEELRQGLSGGGGSLASGGPGPGHASLSQRLRLYLADSWNQCDLVALTCFLLGVGCRLTPGLYHLGRTVLCII
FTVRLLHIFTVNKQLGPKIVIVSKMMKDVFFFLFFLGVWLVAYGVATEGLLRPRDSDFPSILRRVFYRPYLQIFGQIPQEDMI
MEHSNCSSEPGFWAHPPGAQAGTCVSQYANWLVVLLLVIFLLVANILLVNLLIAMFSYTFGKVQGNSDLYWKAQRYRLIREFF
ALAPPFIVISHLRLLLRQLCRRPRSPQPSSPALEHFRVYLSKEAERKLLTWESVHKENFLLARARDKRESDSERLKRTSQKVI
KQLGHIREYEQRLKVLEREVQQCSRVLGWVAEALSRSALLPPGGPPPPDLPGSKD
```

B.)

```
        10                  30                  50
ATCCAATGGCGGTCCTTCCATCTCGAAGCTTCCCTCATGGACGCCCTGCTGAATGACCGG
        70                  90                  110
CCTGAGTTCGTGCGCTTGCTCATTTCCCACGGCCTCAGCCTGGGCCACTTCCTGACCCCG
       130                 150                 170
ATGCGCCTGGCCCAACTCTACAGCGCGGCGCCCTCCAACTCGCTCATCCGCAACCTTTTG
       190                 210                 230
GACCAGGCGTCCCACAGCGCAGGCACCAAAGCCCCAGCCCTAAAAGGGGGAGCTGCGGAG
       250                 270                 290
CTCCGGCCCCCTGACGTGGGGCATGTGCTGAGGATGCTGCTGGGGAAGATGTGCGCGCCG
       310                 330                 350
AGATGTATCTGCTCTCGGACAAGGCCACCTCGCCGCTCTCGCTGGATGCTGGCCTCGGGC
  M  Y  L  L  S  D  K  A  T  S  P  L  S  L  D  A  G  L  G  Q
       370                 390                 410
AGGCCCCCTGGAGCGACCTGCTTCTTTGGGCACTGTTGCTGAACAGGGCACAGATGGCCA
  A  P  W  S  D  L  L  L  W  A  L  L  L  N  R  A  Q  M  A  M
       430                 450                 470
TGTACTTCTGGGAGATGGGTTCCAATGCAGTTTCCTCAGCTCTTGGGGCCTGTTTGCTGC
  Y  F  W  E  M  G  S  N  A  V  S  S  A  L  G  A  C  L  L  L
```

Fig. 9 / continuation 4

```
      490                 510                 530
TCCGGGTGATGGCACGCCTGGAGCCTGACGCTGAGGAGGCAGCACGGAGGAAAGACCTGG
  R  V  M  A  R  L  E  P  D  A  E  E  A  A  R  R  K  D  L  A
      550                 570                 590
CGTTCAAGTTTGAGGGGATGGGCGTTGACCTCTTTGGCGAGTGCTATCGCAGCAGTGAGG
  F  K  F  E  G  M  G  V  D  L  F  G  E  C  Y  R  S  S  E  V
      610                 630                 650
TGAGGGCTGCCCGCCTCCTCCTCCGTCGCTGCCCGCTCTGGGGGGATGCCACTTGCCTCC
  R  A  A  R  L  L  L  R  R  C  P  L  W  G  D  A  T  C  L  Q
      670                 690                 710
AGCTGGCCATGCAAGCTGACGCCCGTGCCTTCTTTGCCCAGGATGGGGTACAGTCTCTGC
  L  A  M  Q  A  D  A  R  A  F  F  A  Q  D  G  V  Q  S  L  L
      730                 750                 770
TGACACAGAAGTGGTGGGGAGATATGGCCAGCACTACACCCATCTGGGCCCTGGTTCTCG
  T  Q  K  W  W  G  D  M  A  S  T  T  P  I  W  A  L  V  L  A
      790                 810                 830
CCTTCTTTTGCCCTCCACTCATCTACACCCGCCTCATCACCTTCAGGAAATCAGAAGAGG
  F  F  C  P  P  L  I  Y  T  R  L  I  T  F  R  K  S  E  E  E
      850                 870                 890
AGCCCACACGGGAGGAGCTAGAGTTTGACATGGATAGTGTCATTAATGGGGAAGGGCCTG
  P  T  R  E  E  L  E  F  D  M  D  S  V  I  N  G  E  G  P  V
      910                 930                 950
TCGGGACGGCGGACCCAGCCGAGAAGACGCCGCTGGGGGTCCCGCGCCAGTCGGGCCGTC
  G  T  A  D  P  A  E  K  T  P  L  G  V  P  R  Q  S  G  R  P
      970                 990                1010
CGGGTTGCTGCGGGGGCCGCTGCGGGGGGCGCCGGTGCCTACGCCGCTGGTTCCACTTCT
  G  C  C  G  G  R  C  G  G  R  R  C  L  R  R  W  F  H  F  W
     1030                1050                1070
GGGGCGTGCCGGTGACCATCTTCATGGGCAACGTGGTCAGCTACCTGCTGTTCCTGCTGC
  G  V  P  V  T  I  F  M  G  N  V  V  S  Y  L  L  F  L  L  L
     1090                1110                1130
TTTTCTCGCGGGTGCTGCTCGTGGATTTCCAGCCGGCGCCGCCCGGCTCCCTGGAGCTGC
  F  S  R  V  L  L  V  D  F  Q  P  A  P  P  G  S  L  E  L  L
     1150                1170                1190
TGCTCTATTTCTGGGCTTTCACGCTGCTGTGCGAGGAACTGCGCCAGGGCCTGAGCGGAG
  L  Y  F  W  A  F  T  L  L  C  E  E  L  R  Q  G  L  S  G  G
     1210                1230                1250
GCGGGGGCAGCCTCGCCAGCGGGGGCCCCGGGCCTGGCCATGCCTCACTGAGCCAGCGCC
  G  G  S  L  A  S  G  G  P  G  P  G  H  A  S  L  S  Q  R  L
     1270                1290                1310
TGCGCCTCTACCTCGCCGACAGCTGGAACCAGTGCGACCTAGTGGCTCTCACCTGCTTCC
  R  L  Y  L  A  D  S  W  N  Q  C  D  L  V  A  L  T  C  F  L
     1330                1350                1370
TCCTGGGCGTGGGCTGCCGGCTGACCCCGGGTTTGTACCACCTGGGCCGCACTGTCCTCT
  L  G  V  G  C  R  L  T  P  G  L  Y  H  L  G  R  T  V  L  C
     1390                1410                1430
GCATCGACTTCATGGTTTTCACGGTGCGGCTGCTTCACATCTTCACGGTCAACAAACAGC
  I  D  F  M  V  F  T  V  R  L  L  H  I  F  T  V  N  K  Q  L
     1450                1470                1490
TGGGGCCCAAGATCGTCATCGTGAGCAAGATGATGAAGGACGTGTTCTTCTTCCTCTTCT
  G  P  K  I  V  I  V  S  K  M  M  K  D  V  F  F  F  L  F  F
     1510                1530                1550
TCCTCGGCGTGTGGCTGGTAGCCTATGGCGTGGCCACGGAGGGGCTCCTGAGGCCACGGG
  L  G  V  W  L  V  A  Y  G  V  A  T  E  G  L  L  R  P  R  D
     1570                1590                1610
ACAGTGACTTCCCAAGTATCCTGCGCCGCGTCTTCTACCGTCCCTACCTGCAGATCTTCG
  S  D  F  P  S  I  L  R  R  V  F  Y  R  P  Y  L  Q  I  F  G
     1630                1650                1670
GGCAGATTCCCCAGGAGGACATGGACGTGGCCCTCATGGAGCACAGCAACTGCTCGTCGG
  Q  I  P  Q  E  D  M  D  V  A  L  M  E  H  S  N  C  S  S  E
     1690                1710                1730
AGCCCGGCTTCTGGGCACACCCTCCTGGGGCCCAGGCGGGCACCTGCGTCTCCCAGTATG
```

Fig. 9 / continuation 5

```
  P  G  F  W  A  H  P  P  G  A  Q  A  G  T  C  V  S  Q  Y  A
     1750                 1770                 1790
CCAACTGGCTGGTGGTGCTGCTCCTCGTCATCTTCCTGCTCGTGGCCAACATCCTGCTGG
  N  W  L  V  V  L  L  L  V  I  F  L  L  V  A  N  I  L  L  V
     1810                 1830                 1850
TCAACTTGCTCATTGCCATGTTCAGTTACACATTCGGCAAAGTACAGGGCAACAGCGATC
  N  L  L  I  A  M  F  S  Y  T  F  G  K  V  Q  G  N  S  D  L
     1870                 1890                 1910
TCTACTGGAAGGCGCAGCGTTACCGCCTCATCCGGGAATTCCACTCTCGGCCCGCGCTGG
  Y  W  K  A  Q  R  Y  R  L  I  R  E  F  H  S  R  P  A  L  A
     1930                 1950                 1970
CCCCGCCCTTTATCGTCATCTCCCACTTGCGCCTCCTGCTCAGGCAATTGTGCAGGCGAC
  P  P  F  I  V  I  S  H  L  R  L  L  L  R  Q  L  C  R  R  P
     1990                 2010                 2030
CCCGGAGCCCCCAGCCGTCCTCCCCGGCCCTCGAGCATTTCCGGGTTTACCTTTCTAAGG
  R  S  P  Q  P  S  S  P  A  L  E  H  F  R  V  Y  L  S  K  E
     2050                 2070                 2090
AAGCCGAGCGGAAGCTGCTAACGTGGGAATCGGTGCATAAGGAGAACTTTCTGCTGGCAC
  A  E  R  K  L  L  T  W  E  S  V  H  K  E  N  F  L  L  A  R
     2110                 2130                 2150
GCGCTAGGGACAAGCGGGAGAGCGACTCCGAGCGTCTGAAGCGCACGTCCCAGAAGGTGG
  A  R  D  K  R  E  S  D  S  E  R  L  K  R  T  S  Q  K  V  D
     2170                 2190                 2210
ACTTGGCACTGAAACAGCTGGGACACATCCGCGAGTACGAACAGCGCCTGAAAGTGCTGG
  L  A  L  K  Q  L  G  H  I  R  E  Y  E  Q  R  L  K  V  L  E
     2230                 2250                 2270
AGCGGGAGGTCCAGCAGTGTAGCCGCGTCCTGGGGTGGGTGGCCGAGGCCCTGAGCCGCT
  R  E  V  Q  Q  C  S  R  V  L  G  W  V  A  E  A  L  S  R  S
     2290                 2310                 2330
CTGCCTTGCTGCCCCCAGGTGGGCCGCCACCCCCTGACCTGCCTGGGTCCAAAGACTGAG
  A  L  L  P  P  G  G  P  P  P  P  D  L  P  G  S  K  D  *
     2350                 2370                 2390
CCCTGCTGGCGGACTTCAAGGAGAAGCCCCCACAGGGGATTTTGCTCCTAGAGTAAGGCT
     2410                 2430                 2450
CATCTGGGCCTCGGCCCCCGCACCTGGTGGCCTTGTCCTTGAGGTGAGCCCCATGTCCAT
     2470                 2490                 2510
CTGGGCCACTGTCAGGACCACCTTTGGGAGTGTCATCCTTACAAACCACAGCATGCCCGG
     2530                 2550                 2570
CTCCTCCCAGAACCAGTCCCAGCCTGGGAGGATCAAGGCCTGGATCCCGGGCCGTTATCC
     2590                 2610                 2630
ATCTGGAGGCTGCAGGGTCCTTGGGGTAACAGGGACCACAGACCCCTCACCACTCACAGA
     2650                 2670                 2690
TTCCTCACACTGGGGAAATAAAGCCATTTCAGAGGAAAAAAAAAAAAAAAAAAAA
```

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MYLLSDKATSPLSLDAGLGQAPWSDLLLWALLLNRAQMAMYFWEMGSNAVSSALGACLLLRVMARLEPDAEEAARRKDLAFKFEGM
GVDLFGECYRSSEVRAARLLLRRCPLWGDATCLQLAMQADARAFFAQDGVQSLLTQKWWGDMASTTPIWALVLAFFCPPLIYTRLI
TFRKSEEEPTREELEFDMDSVINGEGPVGTADPAEKTPLGVPRQSGRPGCCGGRCGGRRCLRRWFHFWGVPVTIFMGNVVSYLLFL
LLFSRVLLVDFQPAPPGSLELLLYFWAFTLLCEELRQGLSGGGGSLASGGPGPGHASLSQRLRLYLADSWNQCDLVALTCFLLGVG
CRLTPGLYHLGRTVLCIDFMVFTVRLLHIFTVNKQLGPKIVIVSKMMKDVFFFLFFLGVWLVAYGVATEGLLRPRDSDFPSILRRV
FYRPYLQIFGQIPQEDMDVALMEHSNCSSEPGFWAHPPGAQAGTCVSQYANWLVVLLLVIFLLVANILLVNLLIAMFSYTFGKVQG
NSDLYWKAQRYRLIREFHSRPALAPPFIVISHLRLLLRQLCRRPRSPQPSSPALEHFRVYLSKEAERKLLTWESVHKENFLLARAR
DKRESDSERLKRTSQKVDLALKQLGHIREYEQRLKVLEREVQQCSRVLGWVAEALSRSALLPPGGPPPPDLPGSKD
```

Fig. 10

A)

```
        10                  30                  50
ATTAAAGTTTATAAAACAGTGGCTGGATGGTTGGAGGATGCAGGTGGACAGAAGACGTGG
                          M  V  G  G  C  R  W  T  E  D  V  E
        70                  90                 110
AGCCTGCAGAAGTAAAGGAAAAGATGTCCTTTCGGGCAGCCAGGCTCAGCATGAGGAACA
  P  A  E  V  K  E  K  M  S  F  R  A  A  R  L  S  M  R  N  R
       130                 150                 170
GAAGGAATGACACTCTGGACAGCACCCGGACCCTGTACTCCAGCGCGTCTCGGAGCACAG
  R  N  D  T  L  D  S  T  R  T  L  Y  S  S  A  S  R  S  T  D
       190                 210                 230
ACTTGTCTTACAGTGAAAGCGCCAGCTTCTACGCTGCCTTCAGGACACAGACGTGCCCAA
  L  S  Y  S  E  S  A  S  F  Y  A  A  F  R  T  Q  T  C  P  I
       250                 270                 290
TCATGGCTTCTTGGGACTTGGTGAATTTTATTCAAGCAAATTTTAAGAAACGAGAATGTG
  M  A  S  W  D  L  V  N  F  I  Q  A  N  F  K  K  R  E  C  V
       310                 330                 350
TCTTCTTTACCAAAGATTCCAAGGCCACGGAGAATGTGTGCAAGTGTGGCTATGCCCAGA
  F  F  T  K  D  S  K  A  T  E  N  V  C  K  C  G  Y  A  Q  S
       370                 390                 410
GCCAGCACATGGAAGGCACCCAGATCAACCAAAGTGAGAAATGGAACTACAAGAAACACA
  Q  H  M  E  G  T  Q  I  N  Q  S  E  K  W  N  Y  K  K  H  T
       430                 450                 470
CCAAGGAATTTCCTACCGACGCCTTTGGGGATATTCAGTTTGAGACACTGGGGAAGAAAG
  K  E  F  P  T  D  A  F  G  D  I  Q  F  E  T  L  G  K  K  G
       490                 510                 530
GGAAGTATATACGTCTGTCCTGCGACACGGACGCGGAAATCCTTTACGAGCTGCTGACCC
  K  Y  I  R  L  S  C  D  T  D  A  E  I  L  Y  E  L  L  T  Q
       550                 570                 590
AGCACTGGCACCTGAAAACACCCAACCTGGTCATTTCTGTGACCGGGGGCGCCAAGAACT
  H  W  H  L  K  T  P  N  L  V  I  S  V  T  G  G  A  K  N  F
       610                 630                 650
TCGCCCTGAAGCCGCGCATGCGCAAGATCTTCAGCCGGCTCATCTACATCGCGCAGTCCA
  A  L  K  P  R  M  R  K  I  F  S  R  L  I  Y  I  A  Q  S  K
       670                 690                 710
AAGGTGCTTGGATTCTCACGGGAGGCACCCATTATGGCCTGATGAAGTACATCGGGGAGG
  G  A  W  I  L  T  G  G  T  H  Y  G  L  M  K  Y  I  G  E  V
       730                 750                 770
TGGTGAGAGATAACACCATCAGCAGGAGTTCAGAGGAGAATATTGTGGCCATTGGCATAG
  V  R  D  N  T  I  S  R  S  S  E  E  N  I  V  A  I  G  I  A
       790                 810                 830
CAGCTTGGGGCATGGTCTCCAACCGGGACACCCTCATCAGGAATTGCGATGCTGAGGGCT
  A  W  G  M  V  S  N  R  D  T  L  I  R  N  C  D  A  E  G  Y
       850                 870                 890
ATTTTTTAGCCCAGTACCTTATGGATGACTTCACAAGAGATCCACTGTATATCCTGGACA
  F  L  A  Q  Y  L  M  D  D  F  T  R  D  P  L  Y  I  L  D  N
       910                 930                 950
ACAACCACACACATTTGCTGCTCGTGGACAATGGCTGTCATGGACATCCCACTGTCGAAG
  N  H  T  H  L  L  L  V  D  N  G  C  H  G  H  P  T  V  E  A
       970                 990                1010
CAAAGCTCCGGAATCAGCTAGAGAAGTATATCTCTGAGCGCACTATTCAAGATTCCAACT
  K  L  R  N  Q  L  E  K  Y  I  S  E  R  T  I  Q  D  S  N  Y
      1030                1050                1070
ATGGTGGCAAGATCCCCATTGTGTGTTTTGCCCAAGGAGGTGGAAAAGAGACTTTGAAAG
  G  G  K  I  P  I  V  C  F  A  Q  G  G  G  K  E  T  L  K  A
      1090                1110                1130
CCATCAATACCTCCATCAAAAATAAAATTCCTTGTGTGGTGGTGGAAGGCTCGGGCCAGA
  I  N  T  S  I  K  N  K  I  P  C  V  V  V  E  G  S  G  Q  I
      1150                1170                1190
TCGCTGATGTGATCGCTAGCCTGGTGGAGGTGGAGGATGCCCTGACATCTTCTGCCGTCA
  A  D  V  I  A  S  L  V  E  V  E  D  A  L  T  S  S  A  V  K
      1210                1230                1250
```

Fig. 10 / continuation 1

```
AGGAGAAGCTGGTGCGCTTTTTACCCCGCACGGTGTCCCGGCTGCCTGAGGAGGAGACTG
  E  K  L  V  R  F  L  P  R  T  V  S  R  L  P  E  E  E  T  E
     1270                 1290                 1310
AGAGTTGGATCAAATGGCTCAAAGAAATTCTCGAATGTTCTCACCTATTAACAGTTATTA
  S  W  I  K  W  L  K  E  I  L  E  C  S  H  L  L  T  V  I  K
     1330                 1350                 1370
AAATGGAAGAAGCTGGGGATGAAATTGTGAGCAATGCCATCTCCTACGCTCTATACAAAG
  M  E  E  A  G  D  E  I  V  S  N  A  I  S  Y  A  L  Y  K  A
     1390                 1410                 1430
CCTTCAGCACCAGTGAGCAAGACAAGGATAACTGGAATGGGCAGCTGAAGCTTCTGCTGG
  F  S  T  S  E  Q  D  K  D  N  W  N  G  Q  L  K  L  L  L  E
     1450                 1470                 1490
AGTGGAACCAGCTGGACTTAGCCAATGATGAGATTTTCACCAATGACCGCCGATGGGAGA
  W  N  Q  L  D  L  A  N  D  E  I  F  T  N  D  R  R  W  E  K
     1510                 1530                 1550
AGAGCAAACCGAGGCTCAGAGACACAATAATCCAGGTCACATGGCTGGAAAATGGTAGAA
  S  K  P  R  L  R  D  T  I  I  Q  V  T  W  L  E  N  G  R  I
     1570                 1590                 1610
TCAAGGTTGAGAGCAAAGATGTGACTGACGGCAAAGCCTCTTCTCATATGCTGGTGGTTC
  K  V  E  S  K  D  V  T  D  G  K  A  S  S  H  M  L  V  V  L
     1630                 1650                 1670
TCAAGTCTGCTGACCTTCAAGAAGTCATGTTTACGGCTCTCATAAAGGACAGACCCAAGT
  K  S  A  D  L  Q  E  V  M  F  T  A  L  I  K  D  R  P  K  F
     1690                 1710                 1730
TTGTCCGCCTCTTTCTGGAGAATGGCTTGAACCTACGGAAGTTTCTCACCCATGATGTCC
  V  R  L  F  L  E  N  G  L  N  L  R  K  F  L  T  H  D  V  L
     1750                 1770                 1790
TCACTGAACTCTTCTCCAACCACTTCAGCACGCTTGTGTACCGGAATCTGCAGATCGCCA
  T  E  L  F  S  N  H  F  S  T  L  V  Y  R  N  L  Q  I  A  K
     1810                 1830                 1850
AGAATTCCTATAATGATGCCCTCCTCACGTTTGTCTGGAAACTGGTTGCGAACTTCCGAA
  N  S  Y  N  D  A  L  L  T  F  V  W  K  L  V  A  N  F  R  R
     1870                 1890                 1910
GAGGCTTCCGGAAGGAAGACAGAAATGGCCGGGACGAGATGGACATAGAACTCCACGACG
  G  F  R  K  E  D  R  N  G  R  D  E  M  D  I  E  L  H  D  V
     1930                 1950                 1970
TGTCTCCTATTACTCGGCACCCCCTGCAAGCTCTCTTCATCTGGGCCATTCTTCAGAATA
  S  F  I  T  R  H  P  L  Q  A  L  F  I  W  A  I  L  Q  N  K
     1990                 2010                 2030
AGAAGGAACTCTCCAAAGTCATTTGGGAGCAGACCAGGGGCTGCACTCTGGCAGCCCTGG
  K  E  L  S  K  V  I  W  E  Q  T  R  G  C  T  L  A  A  L  G
     2050                 2070                 2090
GAGCCAGCAAGCTTCTGAAGACTCTGGCCAAAGTGAAGAACGACATCAATGCTGCTGGGG
  A  S  K  L  L  K  T  L  A  K  V  K  N  D  I  N  A  A  G  E
     2110                 2130                 2150
AGTCCGAGGAGCTGGCTAATGAGTACGAGACCCGGGCTGTTGGTGAGTCCACAGTGTGGA
  S  E  E  L  A  N  E  Y  E  T  R  A  V  G  E  S  T  V  W  N
     2170                 2190                 2210
ATGCTGTGGTGGGCGCGGATCTGCCATGTGGCACAGACATTGCCAGCGGCACTCATAGAC
  A  V  V  G  A  D  L  P  C  G  T  D  I  A  S  G  T  H  R  P
     2230                 2250                 2270
CAGATGGTGGAGAGCTGTTCACTGAGTGTTACAGCAGCGATGAAGACTTGGCAGAACAGC
  D  G  G  E  L  F  T  E  C  Y  S  S  D  E  D  L  A  E  Q  L
     2290                 2310                 2330
TGCTGGTCTATTCCTGTGAAGCTTGGGGTGGAAGCAACTGTCTGGAGCTGGCGGTGGAGG
  L  V  Y  S  C  E  A  W  G  G  S  N  C  L  E  L  A  V  E  A
     2350                 2370                 2390
CCACAGACCAGCATTTCATCGCCCAGCCTGGGGTCCAGAATTTTCTTTCTAAGCAATGGT
  T  D  Q  H  F  I  A  Q  P  G  V  Q  N  F  L  S  K  Q  W  Y
     2410                 2430                 2450
ATGGAGAGATTTCCCGAGACACCAAGAACTGGAAGATTATCCTGTGTCTGTTTATTATAC
  G  E  I  S  R  D  T  K  N  W  K  I  I  L  C  L  F  I  I  P
```

Fig. 10 / continuation 2

```
     2470                2490                2510
CCTTGGTGGGCTGTGGCTTTGTATCATTTAGGAAGAAACCTGTCGACAAGCACAAGAAGC
  L  V  G  C  G  F  V  S  F  R  K  K  P  V  D  K  H  K  K  L
     2530                2550                2570
TGCTTTGGTACTATGTGGCGTTCTTCACCTCCCCCTTCGTGGTCTTCTCCTGGAATGTGG
  L  W  Y  Y  V  A  F  F  T  S  P  F  V  V  F  S  W  N  V  V
     2590                2610                2630
TCTTCTACATCGCCTTCCTCCTGCTGTTTGCCTACGTGCTGCTCATGGATTTCCATTCGG
  F  Y  I  A  F  L  L  L  F  A  Y  V  L  L  M  D  F  H  S  V
     2650                2670                2690
TGCCACACCCCCCCGAGCTGGTCCTGTACTCGCTGGTCTTTGTCCTCTTCTGTGATGAAG
  P  H  P  P  E  L  V  L  Y  S  L  V  F  V  L  F  C  D  E  V
     2710                2730                2750
TGAGACAGGGCCGGCCGGCTGCTCCCAGTGCGGGGCCCGCCAAGCCCACGCCCACCCGGA
  R  Q  G  R  P  A  A  P  S  A  G  P  A  K  P  T  P  T  R  N
     2770                2790                2810
ACTCCATCTGGCCCGCAAGCTCCACACGCAGCCCCGGTTCCCGCTCACGCCACTCCTTCC
  S  I  W  P  A  S  S  T  R  S  P  G  S  R  S  R  H  S  F  H
     2830                2850                2870
ACACTTCCCTGCAAGCTGAGGGTGCCAGCTCTGGCCTTGGCCAGCCCAGAAAGGGGTGGA
  T  S  L  Q  A  E  G  A  S  S  G  L  G  Q  P  R  K  G  W  T
     2890                2910                2930
CATTTAAAAATCTGGAAATGGTTGATATTTCCAAGCTGCTGATGTCCCTCTCTGTCCCTT
  F  K  N  L  E  M  V  D  I  S  K  L  L  M  S  L  S  V  P  F
     2950                2970                2990
TCTGTACGCAGTGGTACGTAAATGGGGTGAATTATTTTACTGACCTGTGGAATGTGATGG
  C  T  Q  W  Y  V  N  G  V  N  Y  F  T  D  L  W  N  V  M  D
     3010                3030                3050
ACACGCTGGGGCTTTTTTACTTCATAGCAGGAATTGTATTTCGGCAAGGGATCCTTAGGC
  T  L  G  L  F  Y  F  I  A  G  I  V  F  R  Q  G  I  L  R  Q
     3070                3090                3110
AGAATGAGCAGCGCTGGAGGTGGATATTCCGTTCGGTCATCTACGAGCCCTACCTGGCCA
  N  E  Q  R  W  R  W  I  F  R  S  V  I  Y  E  P  Y  L  A  M
     3130                3150                3170
TGTTCGGCCAGGTGCCCAGTGACGTGGATGGTACCACGTATGACTTTGCCCACTGCACCT
  F  G  Q  V  P  S  D  V  D  G  T  T  Y  D  F  A  H  C  T  F
     3190                3210                3230
TCACTGGGAATGAGTCCAAGCCACTGTGTGTGGAGCTGGATGAGCACAACCTGCCCCGGT
  T  G  N  E  S  K  P  L  C  V  E  L  D  E  H  N  L  P  R  F
     3250                3270                3290
TCCCCGAGTGGATCACCATCCCCCTGGTGTGCATCTACATGTTATCCACCAACATCCTGC
  P  E  W  I  T  I  P  L  V  C  I  Y  M  L  S  T  N  I  L  L
     3310                3330                3350
TGGTCAACCTGCTGGTCGCCATGTTTGGCTACACGGTGGGCACCGTCCAGGAGAACAATG
  V  N  L  L  V  A  M  F  G  Y  T  V  G  T  V  Q  E  N  N  D
     3370                3390                3410
ACCAGGTCTGGAAGTTCCAGAGGTACTTCCTGGTGCAGGAGTACTGCAGCCGCCTCAATA
  Q  V  W  K  F  Q  R  Y  F  L  V  Q  E  Y  C  S  R  L  N  I
     3430                3450                3470
TCCCCTTCCCCTTCATCGTCTTCGCTTACTTCTACATGGTGGTGAAGAAGTGCTTCAAGT
  P  F  P  F  I  V  F  A  Y  F  Y  M  V  V  K  K  C  F  K  C
     3490                3510                3530
GTTGCTGCAAGGAGAAAAACATGGAGTCTTCTGTCTGCTGTGAGTGGTTTATCCATGTGT
  C  C  K  E  K  N  M  E  S  S  V  C  C  E  W  F  I  H  V  Y
     3550                3570                3590
ACTTGGGATCAGAAGCAGCGATTAATTTCAGGGAAGGATGCCTGCATCCAGTGATTGGAA
  L  G  S  E  A  A  I  N  F  R  E  G  C  L  H  P  V  I  G  S
     3610                3630                3650
GCTGGACCCCAGGCTGGCTGGTCTGGACATCCACACGCATTCTCACATGCAGTGCCGGCT
  W  T  P  G  W  L  V  W  T  S  T  R  I  L  T  C  S  A  G  W
     3670                3690                3710
GGCCAGCAGCAGGGAGTCTCAGTGTCACCACACATAGCAGCTGGGTTCCTGCAAAAAGCA
```

Fig. 10 / continuation 3

```
  P  A  A  G  S  L  S  V  T  T  H  S  S  W  V  P  A  K  S  S
     3730                 3750                 3770
GCAAGTCACAGGCCCACCCAGACAGAACGGGTAGAGAATGTGACTCTGCTTCTGGGTGGG
  K  S  Q  A  H  P  D  R  T  G  R  E  C  D  S  A  S  G  W  E
     3790                 3810                 3830
AAGGACAGCCTGCCCGGTGGGTGGAAGAATCCGTGGCCCTGTTTGGCCATCGTGGCCCTG
  G  Q  P  A  R  W  V  E  E  S  V  A  L  F  G  H  R  G  P  V
     3850                 3870                 3890
TTTGGCCACCTACCACTCTAGGCATCACTGAGCTGAATGCGCCGGTCCTCTGA
  W  P  P  T  T  L  G  I  T  E  L  N  A  P  V  L  *
```

MVGGCRWTEDVEPAEVKEKMSFRAARLSMRNRRNDTLDSTRTLYSSASRSTDLSYSESASFYAAFRTQTCPIMASWDLVNFIQANF
KKRECVFFTKDSKATENVCKCGYAQSQHMEGTQINQSEKWNYKKHTKEFFTDAFGDIQFETLGKKGKYIRLSCDTDAEILYELLTQ
HWHLKTPNLVISVTGGAKNFALKPRMRKIFSRLIYIAQSKGAWILTGGTHYGLMKYIGEVVRDNTISRSSEENIVAIGIAAWGMVS
NRDTLIRNCDAEGYFLAQYLMDDFTRDPLYILDNNHTHLLLVDNGCHGHPTVEAKLRNQLEKYISERTIQDSNYGGKIPIVCFAQG
GGKETLKAINTSIKNKIPCVVVEGSGQIADVIASLVEVEDALTSSAVKEKLVRFLPRTVSRLPEEETESWIKWLKEILECSHLLTV
IKMEEAGDEIVSNAISYALYKAFSTSEQDKDNWNGQLKLLLEWNQLDLANDEIFTNDRRWEKSKPRLRDTIIQVTWLENGRIKVES,
KDVTDGKASSHMLVVLKSADLQEVMFTALIKDRPKFVRLFLENGLNLRKFLTHDVLTELFSNHFSTLVYRNLQIAKNSYNDALLTF
VWKLVANFRRGFRKEDRNGRDEMDIELHDVSPITRHPLQALFIWAILQNKKELSKVIWEQTRGCTLAALGASKLLKTLAKVKNDIN
AAGESEELANEYETRAVGESTVWNAVVGADLPCGTDIASGTHRPDGGELFTECYSSDEDLAEQLLVYSCEAWGGSNCLELAVEATD
QHFIAQPGVQNFLSKQWYGEISRDTKNWKIILCLFIIPLVGCGFVSFRKKPVDKHKKLLWYYVAFFTSPFVVFSWNVVFYIAFLLL
FAYVLLMDFHSVPHPPELVLYSLVFVLFCDEVRQGRPAAPSAGPAKPTPTRNSIWPASSTRSPGSRSRHSFHTSLQAEGASSGLGQ
PRKGWTFKNLEMVDISKLLMSLSVPFCTQWYVNGVNYFTDLWNVMDTLGLFYFIAGIVFRQGILRQNEQRWRWIFRSVIYEPYLAM
FGQVPSDVDGTTYDFAHCTFTGNESKPLCVELDEHNLPRFPEWITIPLVCIYMLSTNILLVNLLVAMFGYTVGTVQENNDQVWKFQ
RYFLVQEYCSRLNIPFPFIVFAYFYMVVKKCFKCCCKEKNMESSVCCEWFIHVYLGSEAAINFREGCLHPVIGSWTPGWLVWTSTR
ILTCSAGWPAAGSLSVTTHSSWVPAKSSKSQAHPDRTGRECDSASGWEGQPARWVEESVALFGHRGPVWPPTTLGITELNAPVL

B.

```
                                                       Q  L
     2290                 2310                 2330
TGCTGGTCTATTCCTGTGAAGCTTGGGGTGGAAGCAACTGTCTGGAGCTGGCGGTGGAGG
  L  V  Y  S  C  E  A  W  G  G  S  N  C  L  E  L  A  V  E  A
     2350                 2370                 2390
CCACAGACCAGCATTTCATCGCCCAGCCTGGGGTCCAGAATTTTCTTTCTAAGCAATGGT
  T  D  Q  H  F  I  A  Q  P  G  V  Q  N  F  L  S  K  Q  W  Y
     2410                 2430                 2450
ATGGAGAGATTTCCCGAGACACCAAGAACTGGAAGATTATCCTGTGTCTGTTTATTATAC
  G  E  I  S  R  D  T  K  N  W  K  I  I  L  C  L  F  I  I  P
     2470                 2490                 2510
CCTTGGTGGGCTGTGGCTTTGTATCATTTAGGAAGAAACCTGTCGACAAGCACAAGAAGC
  L  V  G  C  G  F  V  S  F  R  K  K  P  V  D  K
```

Figure 11:

a.) Trp10b cDNA and derived amino acid sequence

```
        10                  30                  50
ATGAAATCCTTCCTTCCTGTCCACACCATCGTGCTTATCAGGGAGAATGTGTGCAAGTGT
M  K  S  F  L  P  V  H  T  I  V  L  I  R  E  N  V  C  K  C
        70                  90                 110
GGCTATGCCCAGAGCCAGCACATGGAAGGCACCCAGATCAACCAAAGTGAGAAATGGAAC
G  Y  A  Q  S  Q  H  M  E  G  T  Q  I  N  Q  S  E  K  W  N
       130                 150                 170
TACAAGAAACACACCAAGGAATTTCCTACCGACGCCTTTGGGGATATTCAGTTTGAGACA
Y  K  K  H  T  K  E  F  P  T  D  A  F  G  D  I  Q  F  E  T
       190                 210                 230
CTGGGGAAGAAAGGGAAGTATATACGTCTGTCCTGCGACACGGACGCGGAAATCCTTTAC
L  G  K  K  G  K  Y  I  R  L  S  C  D  T  D  A  E  I  L  Y
       250                 270                 290
GAGCTGCTGACCCAGCACTGGCACCTGAAAACACCCAACCTGGTCATTTCTGTGACCGGG
E  L  L  T  Q  H  W  H  L  K  T  P  N  L  V  I  S  V  T  G
       310                 330                 350
GGCGCCAAGAACTTCGCCCTGAAGCCGCGCATGCGCAAGATCTTCAGCCGGCTCATCTAC
G  A  K  N  F  A  L  K  P  R  M  R  K  I  F  S  R  L  I  Y
       370                 390                 410
ATCGCGCAGTCCAAAGGTGCTTGGATTCTCACGGGAGGCACCCATTATGGCCTGATGAAG
I  A  Q  S  K  G  A  W  I  L  T  G  G  T  H  Y  G  L  M  K
       430                 450                 470
TACATCGGGGAGGTGGTGAGAGATAACACCATCAGCAGGAGTTCAGAGGAGAATATTGTG
Y  I  G  E  V  V  R  D  N  T  I  S  R  S  S  E  E  N  I  V
       490                 510                 530
GCCATTGGCATAGCAGCTTGGGGCATGGTCTCCAACCGGGACACCCTCATCAGGAATTGC
A  I  G  I  A  A  W  G  M  V  S  N  R  D  T  L  I  R  N  C
       550                 570                 590
GATGCTGAGGGCTATTTTTTAGCCCAGTACCTTATGGATGACTTCACAAGAGATCCACTG
D  A  E  G  Y  F  L  A  Q  Y  L  M  D  D  F  T  R  D  P  L
       610                 630                 650
TATATCCTGGACAACAACCACACACATTTGCTGCTCGTGGACAATGGCTGTCATGGACAT
Y  I  L  D  N  N  H  T  H  L  L  L  V  D  N  G  C  H  G  H
       670                 690                 710
CCCACTGTCGAAGCAAAGCTCCGGAATCAGCTAGAGAAGTATATCTCTGAGCGCACTATT
P  T  V  E  A  K  L  R  N  Q  L  E  K  Y  I  S  E  R  T  I
       730                 750                 770
CAAGATTCCAACTATGGTGGCAAGATCCCCATTGTGTGTTTTGCCCAAGGAGGTGGAAAA
Q  D  S  N  Y  G  G  K  I  P  I  V  C  F  A  Q  G  G  G  K
       790                 810                 830
GAGACTTTGAAAGCCATCAATACCTCCATCAAAAATAAAATTCCTTGTGTGGTGGTGGAA
E  T  L  K  A  I  N  T  S  I  K  N  K  I  P  C  V  V  V  E
       850                 870                 890
GGCTCGGGCCAGATCGCTGATGTGATCGCTAGCCTGGTGGAGGTGGAGGATGCCCTGACA
G  S  G  Q  I  A  D  V  I  A  S  L  V  E  V  E  D  A  L  T
       910                 930                 950
TCTTCTGCCGTCAAGGAGAAGCTGGTGCGCTTTTTACCCCGCACGGTGTCCCGGCTGCCT
S  S  A  V  K  E  K  L  V  R  F  L  P  R  T  V  S  R  L  P
       970                 990                1010
GAGGAGGAGACTGAGAGTTGGATCAAATGGCTCAAAGAAATTCTCGAATGTTCTCACCTA
E  E  E  T  E  S  W  I  K  W  L  K  E  I  L  E  C  S  H  L
      1030                1050                1070
TTAACAGTTATTAAAATGGAAGAAGCTGGGGATGAAATTGTGAGCAATGCCATCTCCTAC
L  T  V  I  K  M  E  E  A  G  D  E  I  V  S  N  A  I  S  Y
      1090                1110                1130
GCTCTATACAAAGCCTTCAGCACCAGTGAGCAAGACAAGGATAACTGGAATGGGCAGCTG
A  L  Y  K  A  F  S  T  S  E  Q  D  K  D  N  W  N  G  Q  L
```

Fig. 11 (Continuation)

```
      2410                2430                2450
AGAAACTTAGGACCCAAGATTATAATGCTGCAGAGGATGCTGATCGATGTGTTCTTCTTC
R  N  L  G  P  K  I  I  M  L  Q  R  M  L  I  D  V  F  F  F
      2470                2490                2510
CTGTTCCTCTTTGCGGTGTGGATGGTGGCCTTTGGCGTGGCCAGGCAAGGGATCCTTAGG
L  F  L  F  A  V  W  M  V  A  F  G  V  A  R  Q  G  I  L  R
      2530                2550                2570
CAGAATGAGCAGCGCTGGAGGTGGATATTCCGTTCGGTCATCTACGAGCCCTACCTGGCC
Q  N  E  Q  R  W  R  W  I  F  R  S  V  I  Y  E  P  Y  L  A
      2590                2610                2630
ATGTTCGGCCAGGTGCCCAGTGACGTGGATGGTACCACGTATGACTTTGCCCACTGCACC
M  F  G  Q  V  P  S  D  V  D  G  T  T  Y  D  F  A  H  C  T
      2650                2670                2690
TTCACTGGGAATGAGTCCAAGCCACTGTGTGTGGAGCTGGATGAGCACAACCTGCCCCGG
F  T  G  N  E  S  K  P  L  C  V  E  L  D  E  H  N  L  P  R
      2710                2730                2750
TTCCCCGAGTGGATCACCATCCCCCTGGTGTGCATCTACATGTTATCCACCAACATCCTG
F  P  E  W  I  T  I  P  L  V  C  I  Y  M  L  S  T  N  I  L
      2770                2790                2810
CTGGTCAACCTGCTGGTCGCCATGTTTGGCTACACGGTGGGCACCGTCCAGGAGAACAAT
L  V  N  L  L  V  A  M  F  G  Y  T  V  G  T  V  Q  E  N  N
      2830                2850                2870
GACCAGGTCTGGAAGTTCCAGAGGTACTTCCTGGTGCAGGAGTACTGCAGCCGCCTCAAT
D  Q  V  W  K  F  Q  R  Y  F  L  V  Q  E  Y  C  S  R  L  N
      2890                2910                2930
ATCCCCTTCCCCTTCATCGTCTTCGCTTACTTCTACATGGTGGTGAAGAAGTGCTTCAAG
I  P  F  P  F  I  V  F  A  Y  F  Y  M  V  V  K  K  C  F  K
      2950                2970                2990
TGTTGCTGCAAGGAGAAAAACATGGAGTCTTCTGTCTGCTGTTTCAAAAATGAAGACAAT
C  C  C  K  E  K  N  M  E  S  S  V  C  C  F  K  N  E  D  N
      3010                3030                3050
GAGACTCTGGCATGGGAGGGTGTCATGAAGGAAAACTACCTTGTCAAGATCAACACAAAA
E  T  L  A  W  E  G  V  M  K  E  N  Y  L  V  K  I  N  T  K
      3070                3090                3110
GCCAACGACACCTCAGAGGAAATGAGGCATCGATTTAGACAACTGGATACAAAGCTTAAT
A  N  D  T  S  E  E  M  R  H  R  F  R  Q  L  D  T  K  L  N
      3130                3150
GATCTCAAGGGTCTACTGAAAGAGATTGCTAATAAAATCAAATAG
D  L  K  G  L  L  K  E  I  A  N  K  I  K  *
```

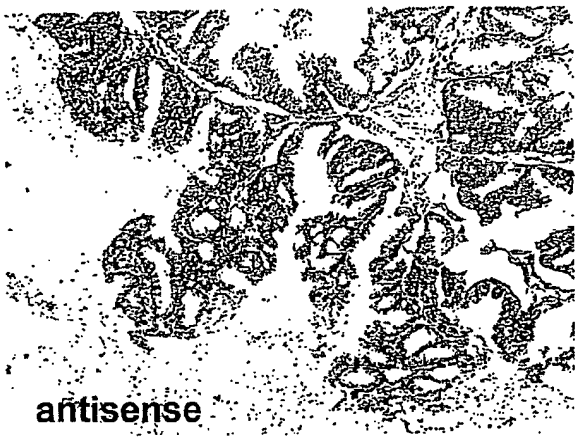
b.) Trp10 protein:

```
MKSFLPVHTIVLIRENVCKCGYAQSQHMEGTQINQSEKWNYKKHTKEFPTDAFGDIQFETLGKKGKYIRLSCDTDAEILY
ELLTQHWHLKTPNLVISVTGGAKNFALKPRMRKIFSRLIYIAQSKGAWILTGGTHYGLMKYIGEVVRDNTISRSSEENIV
AIGIAAWGMVSNRDTLIRNCDAEGYFLAQYLMDDFTRDPLYILDNNHTHLLLVDNGCHGHPTVEAKLRNQLEKYISERTI
QDSNYGGKIPIVCFAQGGGKETLKAINTSIKNKIPCVVVEGSGQIADVIASLVEVEDALTSSAVKEKLVRFLPRTVSRLP
EEETESWIKWLKEILECSHLLTVIKMEEAGDEIVSNAISYALYKAFSTSEQDKDNWNGQLKLLLEWNQLDLANDEIFTND
RRWESADLQEVMFTALIKDRPKFVRLFLENGLNLRKFLTHDVLTELFSNHFSTLVYRNLQIAKNSYNDALLTFVWKLVAN
FRRGFRKEDRNGRDEMDIELHDVSPITRHPLQALFIWAILQNKKELSKVIWEQTRGCTLAALGASKLLKTLAKVKNDINA
AGESEELANEYETRAVELFTECYSSDEDLAEQLLVYSCEAWGGSNCLELAVEATDQHFIAQPGVQNFLSKQWYGEISRDT
KNWKIILCLFIIPLVGCGFVSFRKKPVDKHKKLLWYYVAFFTSPFVVFSWNVVFYIAFLLLFAYVLLMDFHSVPHPPELV
LYSLVFVLFCDEVRQWYVNGVNYFTDLWNVMDTLGLFYFIAGIVFRLHSSNKSSLYSGRVIFCLDYIIFTLRLIHIFTVS
RNLGPKIIMLQRMLIDVFFFLFLPAVWMVAFGVARQGILRQNEQRWRWIFRSVIYEPYLAMFGQVPSDVDGTTYDFAHCT
FTGNESKPLCVELDEHNLPRFPEWITIPLVCIYMLSTNILLVNLLVAMFGYTVGTVQENNDQVWKFQRYFLVQEYCSRLN
IPFPFIVFAYFYMVVKKCFKCCCKEKNMESSVCCFKNEDNETLAWEGVMKENYLVKINTKANDTSEEMRHRFRQLDTKLN
DLKGLLKEIANKIK
```

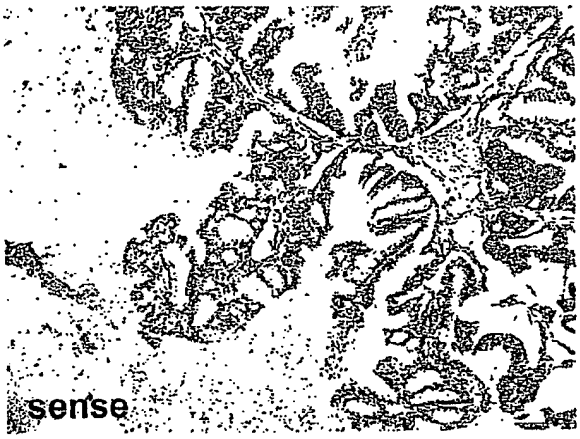
The Trp8 Gene is expre d in endometrial or uteri
in normal endometrium

Endometrial cancer:

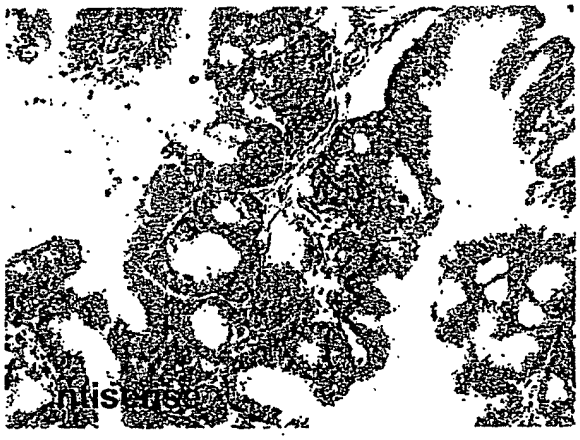
A



B



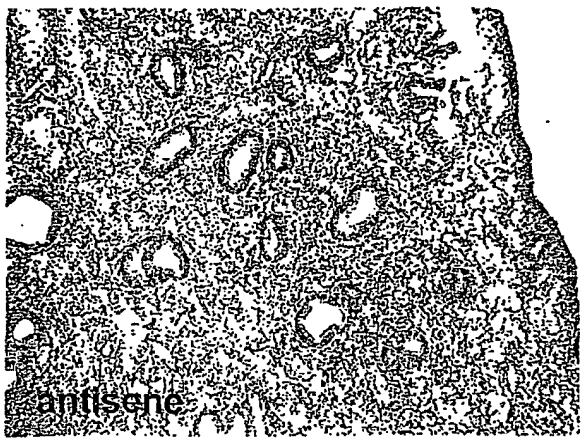
C



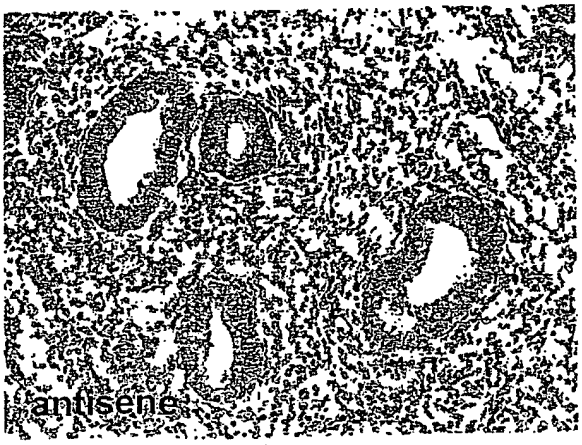
D

Endometrium:

E



F



Expression of human Trp 9 and Trp 10

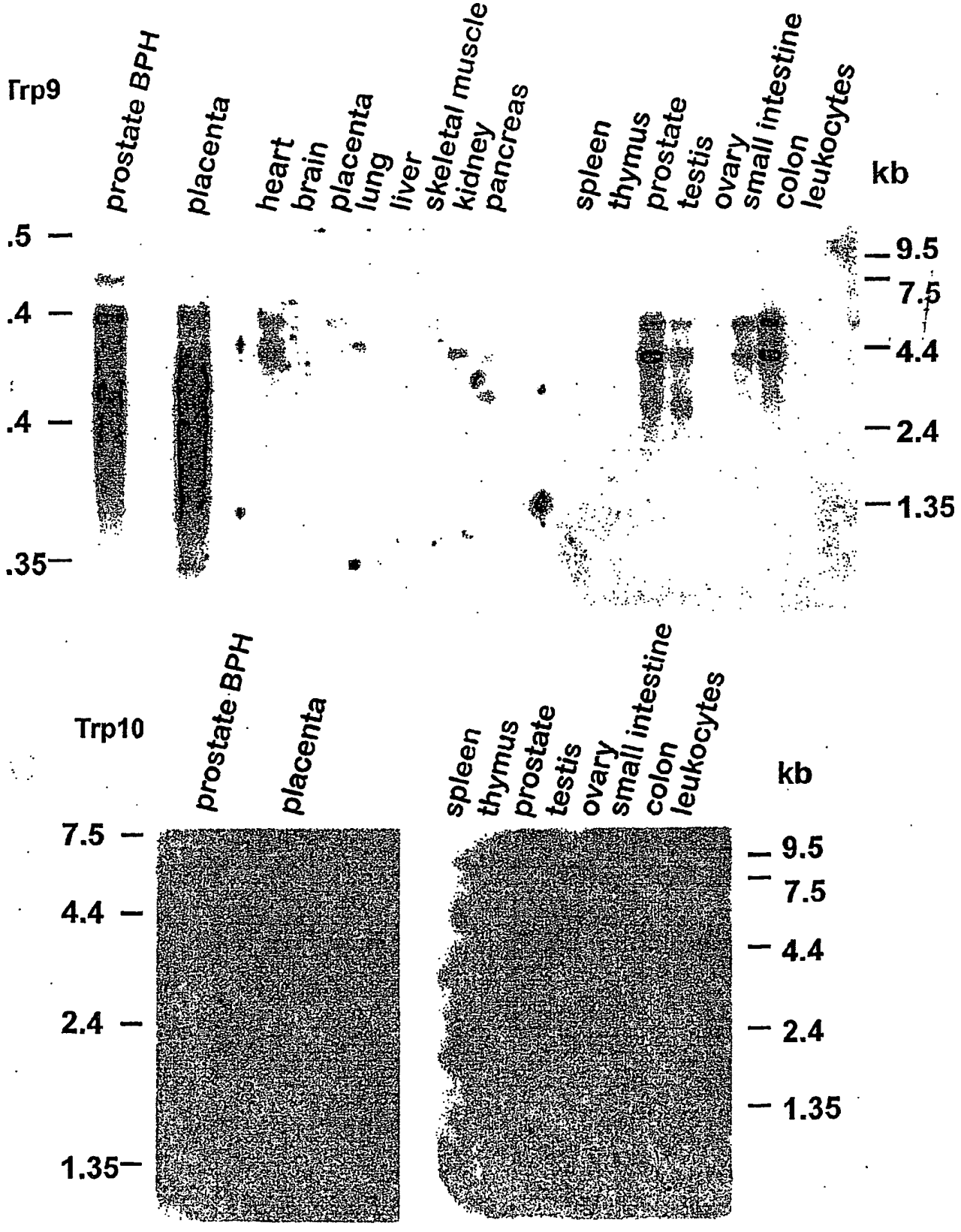
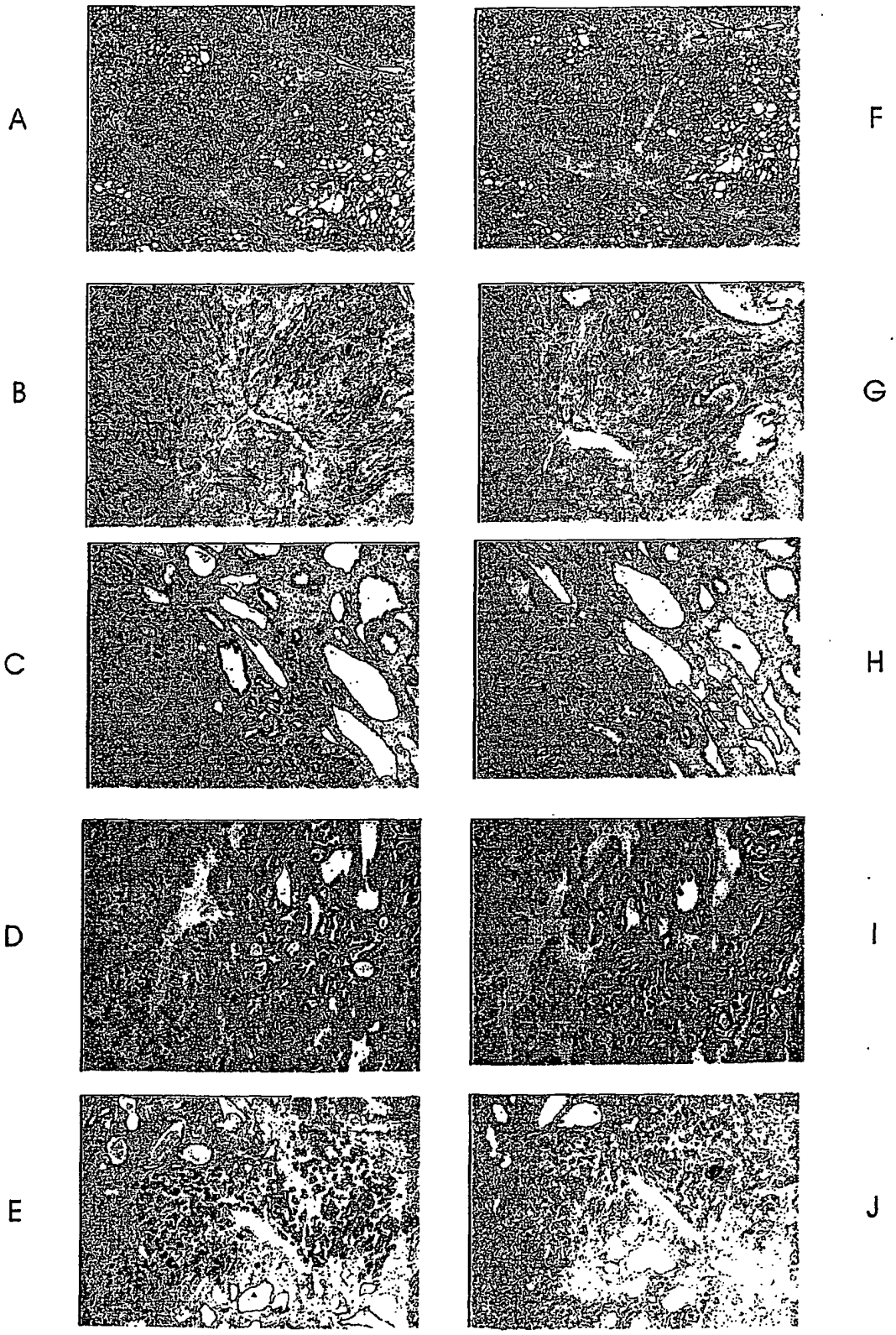
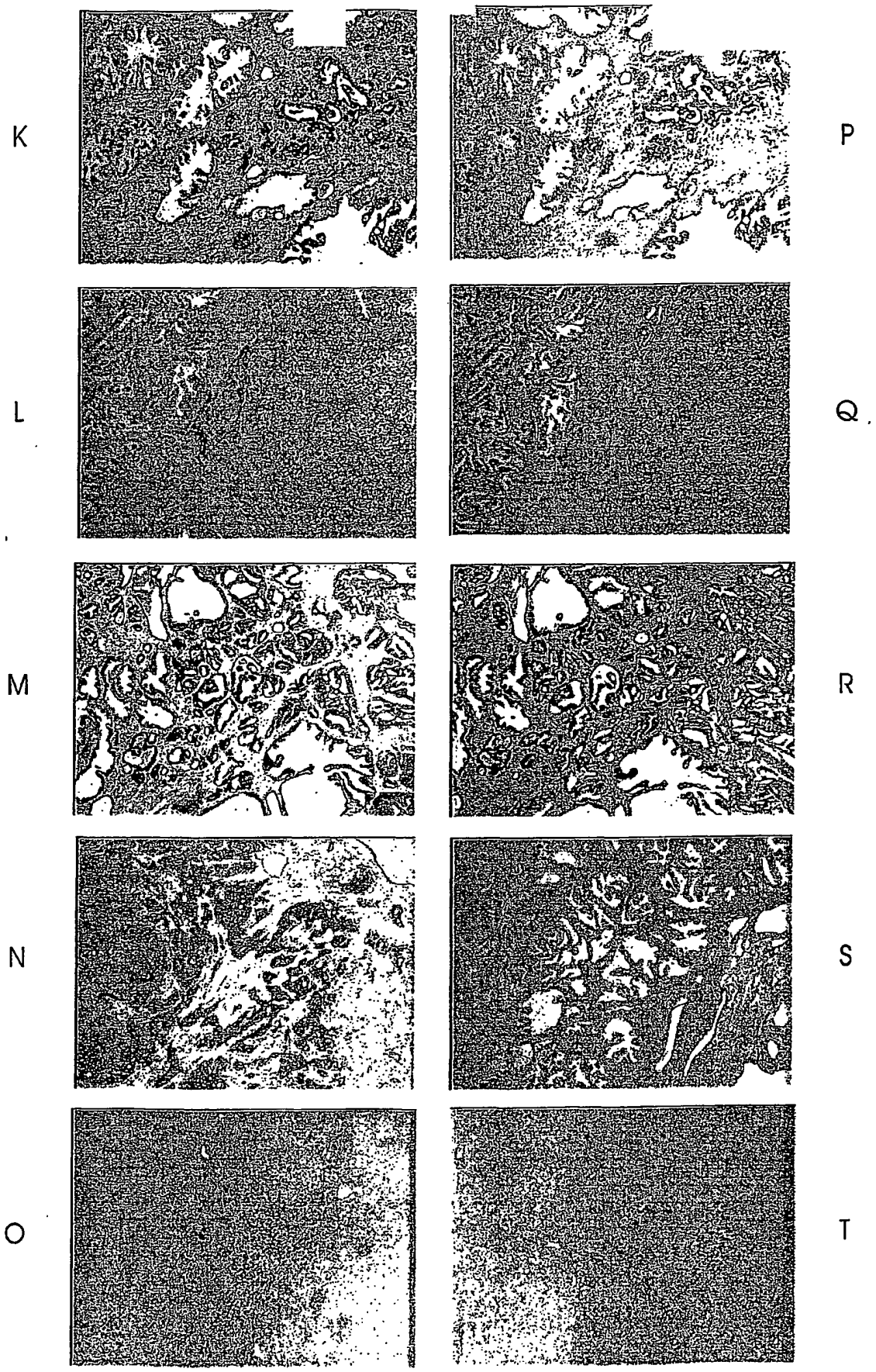


Fig. 14

Expression of Trp10 transcripts and Trp10-antisense transcripts in human prostate cancer and in malignant melanoma

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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(25) Filing Language: English

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60/221,513 28 July 2000 (28.07.2000) US

(71) Applicant and
(72) Inventor: **WISSENBACH, Ulrich** [DE/DE]; Institut für Pharmakologie und Toxikologie der Uni, versität des Saarlandes, 66421 Homburg (DE).

(74) Agent: **HUBER, Bernard**; Huber & Schüssler, Truderinger Str. 246, 81825 München (DE).

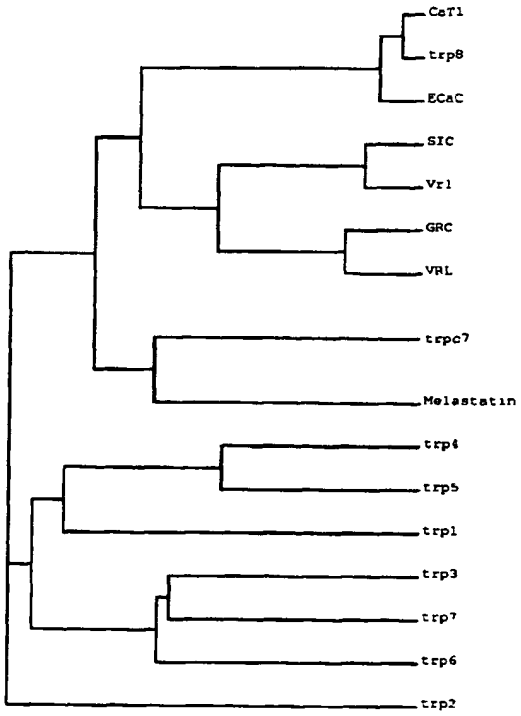
(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

[Continued on next page]

(54) Title: TRP8, TRP9 AND TRP10, MARKERS FOR CANCER



(57) Abstract: The present invention relates to gene expression in normal cells and cells of malignant tumors and particularly to novel markers associated with cancer, Trp8, Trp9 and Trp10, and the genes encoding Trp8, Trp9 and Trp10. Also provided are vectors, host cells, antibodies, and recombinant methods for producing these human proteins. The invention further relates to diagnostic and therapeutic methods useful for diagnosing and treating a tumor.

WO 02/010382 A3

(88) **Date of publication of the international search report:**
9 October 2003

(15) **Information about Correction:**
Previous Correction:
see PCT Gazette No. 38/2002 of 19 September 2002, Section II

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

INTERNATIONAL SEARCH REPORT

rnational Application No
PCT/EP 01/08309

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/12 C12N15/11 C12N9/00 C07K14/47 C12Q1/68
G01N33/577 A61K31/713 C07K14/705

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS, EPO-Internal, SEQUENCE SEARCH, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 09166 A (SHAPERO MICHAEL H ;DENDREON CORP (US); LAUS REINER (US); TSAVALER) 25 February 1999 (1999-02-25) see SEQID14 + 15, pages 2,3, 28,29, Example 4 table 3 ---	1-10, 12-17, 23,29-31
X	WO 00 40614 A (BETH ISRAEL HOSPITAL ;SCHARENBERG ANDREW M (US)) 13 July 2000 (2000-07-13) see seqid31 + 32, page 11, first paragraph, page 44, lines 13-15 --- -/--	1-10,12, 31

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 March 2003	13. 03. 2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Holtorf, S

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MULLER D ET AL: "Molecular cloning, tissue distribution, and chromosomal mapping of the human epithelial Ca2+ channel (ECAC1)." GENOMICS, vol. 67, no. 1, 1 July 2000 (2000-07-01), pages 48-53, XP002222953 ISSN: 0888-7543 the whole document	1
X	WO 98 15657 A (ABBOTT LAB) 16 April 1998 (1998-04-16) the whole document	1-12, 29-31
X	WO 98 37093 A (CORIXA CORP) 27 August 1998 (1998-08-27) the whole document	1-12, 29-31
A	TSAVALER LARISA ET AL: "TRP-P8, a novel prostate-specific gene, is upregulated in prostate cancer and other malignancies and shares high homology with TRP calcium channel proteins." PROCEEDINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH ANNUAL, no. 41, March 2000 (2000-03), page 694 XP008011242 91st Annual Meeting of the American Association for Cancer Research.;San Francisco, California, USA; April 01-05, 2000, March, 2000 ISSN: 0197-016X the whole document	
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P,X	WO 01 14423 A (SMITHKLINE BEECHAM PLC) 1 March 2001 (2001-03-01) see SEQid1 + 2; see example 1	1-9,31

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WISSENBACH ULRICH ET AL: "Expression of CaT-like, a novel calcium-selective channel, correlates with the malignancy of prostate cancer." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 276, no. 22, 1 June 2001 (2001-06-01), pages 19461-19468, XP002222954 ISSN: 0021-9258 the whole document	1-9,13, 14, 16-19, 21-23,29
P,X	WO 01 04303 A (HEDIGER MATTHIAS A) 18 January 2001 (2001-01-18) see SEQID1 + 2 the whole document	1-5
P,X	WO 01 42467 A (MILLENNIUM PREDICTIVE MEDICINE) 14 June 2001 (2001-06-14) see SEQID 4615	1
E	WO 01 51633 A (FANGER GARY RICHARD ;HARLOCKER SUSAN L (US); MEAGHER MADELEINE JOY) 19 July 2001 (2001-07-19) see SEQID764, example 3, claims	1
E	WO 02 14361 A (AGENSYS INC) 21 February 2002 (2002-02-21) see SEQID1479, examples 1-4 the whole document	1-10, 13-23
E	WO 02 00722 A (SILOS SANTIAGO INMACULADA ;CURTIS RORY A J (US); MILLENNIUM PHARM) 3 January 2002 (2002-01-03) see SEQID4	1-5
E	WO 01 68857 A (CURTIS RORY A J ;COOK WILLIAM JAMES (US); MILLENNIUM PHARM INC (US) 20 September 2001 (2001-09-20) see SEQID1, examples	1-5
E	WO 01 53348 A (SQUIBB BRISTOL MYERS CO ;GAUGHAN GLEN T (US); RAMANATHAN CHANDRA S) 26 July 2001 (2001-07-26) see SEQID5 the whole document	1
E	WO 01 62794 A (LORA JOSE M ;CURTIS RORY A J (US); GLUCKSMANN MARIA ALEXANDRA (US)) 30 August 2001 (2001-08-30) the whole document	1-9
E	WO 02 30268 A (EOS BIOTECHNOLOGY INC) 18 April 2002 (2002-04-18) see SEQID53	1,6

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	BOEDDING MATTHIAS ET AL: "The recombinant human TRPV6 channel functions as Ca2+ sensor in human embryonic kidney and rat basophilic leukemia cells." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 277, no. 39, 27 September 2002 (2002-09-27), pages 36656-36664, XP002222955 September 27, 2002 ISSN: 0021-9258 the whole document ---	

INTERNATIONAL SEARCH REPORT

nternational application No.
PCT/EP 01/08309

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 24-28 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [X] Claims Nos.: 12 partially
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. [X] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[X] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-12, 29-31 partially, 13-28 completely

Isolated nucleic acid molecules encoding human prostate carcinom associated proteins as characterized by SEQIDs5,45,11,3 and SEQIDs 6,46,12,4, respectively; the recombinant expression of the same in host cells; the isolated proteins as characterized by SEQIDs 6,46,12,4; antisense RNA sequence and ribozyme complementary to said nucleic acid molecules; inhibitor that can suppress the activity of said prostate carcinom associated proteins; method for diagnosing a prostate carcinoma by contacting a sample with a nucleic acid, an antibody or other reagent that reacts with the mRNA of SEQIDs5,45,11,3; method for diagnosing endomertial cancer by contacting a target sample with a nucleic acid, an antibody or other reagent that reacts with the mRNA of SEQIDs5,45,11,3; method for diagnosing a melanoma, chorion carcinoma, cancer of the lung and ot the prostate comprising contacting a target sample with a reagent which detects antisense RNA of SEQIDs 11 and 3; method for preventing prostate tumour, endometrial cancer, choroin carcinoma or cancer of the lung comprising administering an inhibiting reagent of human prostate carcinom associated proteins; diagnostic kit containing an antibody; method for identifyng an agonist or an antagonist of human prostate carcinom associated proteins.

2. Claims: 1-12, 29-31 partially

Isolated nucleic acid molecule encoding human prostate carcinom associated protein as characterized by SEQIDs 7 and SEQIDs 8, respectively; the recombinant expression of the same in host cells; the isolated protein as characterized by SEQIDs 8; antisense RNA sequence and ribozyme complementary to said nucleic acid molecule; inhibitor that can suppress the activity of said prostate carcinom associated protein; diagnostic kit containing an antibody; method for identifyng an agonist or an antagonist of human prostate carcinom associated proteins.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 12 partially

Present claim 12 relates to an inhibitor wich is defined by reference to a desirable characteristic or property, namely suppressing the activity of the protein of claim 6.

The claims cover all inhibitors having this characteristic or property, whereas the application provides only support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for a limited number of such inhibitors.

In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the inhibitors by reference to a result to be achieved.
Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible.

Consequently, the search has been carried out for those parts of the claim 12 which appear to be clear, supported and disclosed, namely those parts relating to the Trp8/10 corresponding antibody , Trp8/10 corresponding antisense construct, a Trp8/10 corresponding ribozyme.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 01/08309

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9909166	A	25-02-1999	US	6194152 B1	27-02-2001
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(54) Title: TRP8, TRP9 AND TRP10, NOVEL MARKERS FOR CANCER

(57) Abstract: The present invention relates to gene expression in normal cells and cells of malignant tumors and particularly to novel markers associated with cancer, Trp8, Trp9 and Trp10, and the genes encoding Trp8, Trp9 and Trp10. Also provided are vectors, host cells, antibodies, and recombinant methods for producing these human proteins. The invention further relates to diagnostic and therapeutic methods useful for diagnosing and treating a tumor.

Trp8, Trp9 and Trp10, novel markers for cancer

FIELD OF THE INVENTION

The present invention relates to gene expression in normal cells and cells of malignant tumors and particularly to novel markers associated with cancer, Trp8, Trp9 and Trp10, and the genes encoding Trp8, Trp9 and Trp10

BACKROUND OF THE TECHNOLOGY

Prostate cancer is one of the most common diseases of older men world wide. Diagnosis and monitoring of prostate cancer is difficult because of the heterogeneity of the disease. For diagnosis different grades of malignancy can be distinguished according to the Gleason-Score Diagnosis. For this diagnosis a prostate tissue sample is taken from the patient by biopsy and the morphology of the tissue is investigated. However, this approach only yields subjective results depending on the experience of the pathologist. For confirmation of these results and for obtaining an early diagnosis an additional diagnostic method can be applied which is based on the detection of a prostate specific antigen (PSA). PSA is assayed in serum samples, blood samples etc. using an anti-PSA-antibody. However, since in principle PSA is also expressed in normal prostate tissue there is a requirement for the definition of a threshold value (about 4 ng/ml PSA) in order to be able to distinguish between normal and malign prostate tissue. Unfortunately, this diagnostic method is quite insensitive and often yields false-positive results. Moreover, by using this diagnostic method any conclusions as regards the grade of malignancy, the progression of the tumor and its potential for metastasizing cannot be drawn. Thus, the use of molecular markers would be helpful to distinguish benign from malign tissue and for grading and staging prostate carcinoma, particularly for patients with metastasizing prostate cancer having a very bad prognosis.

The above discussed limitations and failings of the prior art to provide meaningful specific markers which correlate with the presence of prostate tumors, in particular metastasizing tumors, has created a need for markers which can be used diagnostically, prognostically and therapeutically over the course of this disease. The present invention fulfils such a need by the provision of Tpr8, Trp9 and Trp10 and the genes encoding Trp8, Trp9 and Trp10: The genes encoding Trp8 and Trp10 are expressed in prostate carcinoma and prostatic metastasis, but

not in normal prostate, benign hyperplasia (BHP) and intraepithelial prostatic neoplasia (PIN). Furthermore, expression of Trp10 transcripts is detectable in carcinoma but not in healthy tissue of the lung, the prostate, the placenta and in melanoma.

SUMMARY OF THE INVENTION

The present invention is based on the isolation of genes encoding novel markers associated witha cancer, Trp8, Trp9 and Trp10. The new calcium channel proteins Trp8, Trp9 and Trp10 are members of the trp (transient receptor potential) - family, isolated from human placenta (Trp8a and Trp8b) and humane prostate (Trp9, Trp10a and Trp10b). Trp proteins belong to a steadily growing family of Ca^{2+} selective and non selective ion channels. In the recent years seven Trp proteins (trp1 - trp7) have been identified and suggested to be involved in cation entry, receptor operated calcium entry and pheromone sensory signaling. Structurally related to the trp proteins are the vanilloid receptor (VR1) and the vanilloid like receptor (VRL-1) both involved in nociception triggered by heat. Furthermore, two calcium permeable channels were identified in rat small intestine (CaT1) and rabbit kidney (ECaC). These distantly related channels are suggested to be involved in the uptake of calcium ions from the lumen of the small intestine (CaT1) or in the reuptake of calcium ions in the distal tubule of the kidney (ECaC). Common features or the Trp and related channels are a proposed structure comprising six transmembrane domains including several conserved amino acid motifs. In the present invention the cloning and expression of a CaT1 like calcium channel (Trp8) from human placenta as well as Trp9 and Trp10 (two variants, Trp10a and Trp10b) is described. Two polymorphic variants of the Trp8 cDNA were isolated from placenta (Trp8a and Trp8b). Transient expression of the Trp8b cDNA in HEK (human embryonic kidney) cells results in cytosolic calcium overload implicating that the Trp8 channel is constitutive open in the expression system. Trp8 induces highly calcium selective inward currents in HEK cells. The C -terminus of the Trp8 protein binds calmodulin in a calcium dependent manner. The Trp9 channel is expressed in trophoblasts and syncytiotrophoblasts of placenta and in pancreatic acinar cells. Furthermore, the Trp8 channel is expressed in prostatic carcinoma and prostatic metastases, but not in normal tissue of the prostate. No expression of Trp8 transcripts is detectable in benign prostatic hyperplasia (BPH) or prostatic intraepithelial neoplasia (PIN). Therefore, the Trp8 channel is exclusively expressed in malign prostatic tissues and serves as molecular marker for prostate cancer. From the experimental results it is also apparent that the

modulation of Trp8 and/or Trp10, e.g. the inhibition of expression or activity , is of therapeutic interest, e.g. for the prevention of tumor progression.

The present invention, thus, provides a Trp8, Trp9 and Trp10 protein, respectively, as well as nucleic acid molecule encoding the protein and, moreover, an antisense RNA, a ribozyme and an inhibitor, which allow to inhibit the expression or the activity of Trp8, Trp9 and/or Trp10.

In one embodiment, the present invention provides a diagnostic method for detecting a prostate cancer or endometrial cancer (cancer of the uterus) associated with Trp8 or Trp10 in a tissue of a subject, comprising contacting a sample containing Trp8 and/or Trp10 encoding mRNA with a reagent which detects Trp8 and/or Trp10 or the corresponding mRNA.

In a further embodiment, the present invention provides a diagnostic method for detecting a melanoma, chorion carcinoma, cancer of the lung and of the prostate in a tissue of a subject, comprising contacting a sample with a reagent which detects Trp10a and/or Trp10b antisense transcripts or Trp10a and/or Trp10b related antisense transcripts.

In another embodiment, the present invention provides a method of treating a prostate tumor, carcinoma of the lung, carcinoma of the placenta (chorion carcinoma) or melanoma associated with Trp8 and/or Trp10, comprising administering to a subject with such an disorder a therapeutically effect amount of a reagent which modulates, e.g. inhibits, expression of Trp8 and/or Trp10 or the activity of the protein, e.g. the above described compounds.

Finally, the present invention provides a method of gene therapy comprising introducing into cells of a subject an expression vector comprising a nucleotide sequence encoding the above mentioned antisense RNA or ribozyme, in operable linkage with a promoter.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: A, phylogenetic relationship of trp and related proteins. B, hydropathy plot of the Trp8 protein sequence according to Kyte and Doolittle. C, alignment of Trp8a/b to the epithelial calcium channels ECaC (from rabbit) and Vr1 (from rat). Putative transmembrane domains are underlined.

Figure 2: A, polymorphism of the Trp8 gene. The polymorphic variants Trp8a and Trp8b differ in five base pairs resulting in three amino acid exchanges in the derived protein sequences. Specific primers were derived from the Trp8 gene as indicated by arrows. B, the Trp8a and Trp8b genes are distinguishable by a single restriction site. Genomic fragments of the Trp8 gene can be amplified using specific primers (shown in A). The genomic fragment of the Trp8b gene contains an additional site of the restriction enzyme BSP1286I (B). C, the Trp8 gene is located on chromosome 7. D, genotyping of eleven human subjects. A 458 bp genomic fragment of the Trp8 gene was amplified using specific primers (shown in A) and restricted with BSP1286I. The resulting fragments were analyzed by PAGE electrophoresis.

Figure 3: The Trp8b protein is a calcium selective ion channel. A, representative trace of a pdiTrp8b transfected HEK 293 cell. Trp8b mediated currents are activated by voltage ramps (-100 mV - +100 mV) of 100 msec at -40 mV or +70 mV holding potential. 1, Trp8b currents in the presence at 2mm $[Ca^{2+}]_o$;2, effect of solution switch alone 3, switch to nominal zero calcium solution. B, Trp8b currents in the presence of zero divalent cations. C, current voltage relationship of the currents shown in A. Inset, leak subtracted current. D, current voltage relationship of the current shown in B. E, statistics of representative experiments. Black: Trp8 transfected cells, gray: control cells. Columns from left to right: Trp8 currents at - 40 mV (n =12) and + 70 mV holding potential (n = 12). Trp8 currents in standard bath solution including 120 mM NMDG without sodium (n = 7) and with nominal zero calcium ions (n = 8) or in the presence of 1mM EGTA with zero divalent cations (n = 6). F, representative changes in $[Ca^{2+}]_i$ in Trp8b transfected HEK cells (gray) and controls (black) in the presence or absence of 1mM $[Ca^{2+}]_o$. Inset, relative increase of cytosolic calcium concentration of Trp8b transfected HEK cells, before and after readdition of 1 mM $[Ca^{2+}]_o$ in comparison to control cells.

Figure 4: The C-terminal region of the Trp8 protein binds calmodulin. A, N- and C-terminal fragments of the Trp8 protein used for calmodulin binding studies. B, the Trp8 protein and a truncated Trp8 protein which was in vitro translated after MunI cut of the cDNA, which lacks the C-terminal 32 amino acid residues, were in vitro translated in the presence of ^{35}S-methionine and incubated with calmodulin coupled agarose beads in the presence of 1 mM Ca^{2+} or 2 mM EGTA. C, calmodulin binding to N- and C-terminal fragments of the Trp8protein in the presence of Ca^{2+} (1 mM) or EGTA (2 mM)

Figure 5: Expression pattern of the Trp8 cDNA. A, Northern blots (left panels, Clontech, Palo Alto) were hybridized using a 348 bp NcoI/BamHT fragment of the Trp9 cDNA. The probe hybridizes to mRNA species isolated from the commercial blot, but not to mRNA species isolated from benign prostate hyperplasia (right panel, mRNA isolated from 20 human subjects with benign prostate hyperplasia). B,C, in situ hybridization with biotinylated Trp8 specific oligonucleotides on slides of human tissues. Left column antisense probes, right column sense probes. D, antinsense probes.

Figure 6: Differential expression of Trp8 cDNA in human prostate. A - F, in situ hybridization with prostatic tissues. A, normal prostate, B, primary carcinoma, C, benign hyperplasia, D, rezidive carcinoma, E, prostatic intraepithelial neoplasia, F, lymphnode metastasis of the prostata.

Figure 7: Trp8a cDNA sequence and derived amino acid sequence

Figure 8: A, Trp8b cDNA sequence and derived amino acid sequence
B, cDNA sequence of splice variant 1 (12B1)
C, cDNA sequence of splice variant 2 (17-3)
D, cDNA sequence of splice variant 3 (23A3)
E, cDNA sequence of splice variant 4 (23C3)

Figure 9: A, Trp9 cDNA sequence and derived amino acid sequence B, cDNA sequence of splice variant 15 and derived amino acid sequence. .

Figure 10: A, cDNA sequence of Trp10a and derived amino acid sequence, B, cDNA fragment of Trp10a and derived amino acid sequence.

Figure 11: cDNA sequence of Trp10b and derived amino acid sequence.

Figure 12: Expression of Trp8 mRNA in human endometrial cancer or cancer of the uterus. A - D, in situ hybridization with slides of endometrial cancer hybridized with Trp8 antisense (left column) or sense probes as controls (right column). E - F, Trp8 antisense probes hybridized to slides of normal endometrium. It can be clearly seen no hybridization occurs with normal endometrial tissue.

Figure 13: Expression of human Trp9 and Trp10 genes
Northern blots were hybridized using Trp9 (upper panel) or Trp10 (lower panel) specific probes. Expression of the Trp9 cDNA is detectable in many tissues including human prostate and colon as well as in benign prostatic hyperplasia. Expression of Trp10 cDNA is detectable in human prostate of a commercial northern blot (Clontech, right side). This Northern blot contains prostatic tissue collected from 15 human subjects in the range of 14 - 60 years of age. No expression of Trp10 cDNA was detectable in benign prostatic hyperplasia (left side).

Figure 14: Expression of Trp10 transcripts and Trp10-antisense transcripts in human prostate cancer and metastasis of a melanoma. In situ hybridizations of slides hybridized with Trp10-antisense (A-E, K-N) and Trp10 related sense probes (F-J, P-R). It can clearly be seen that both probes detect the same cancer cells indicating that these cancer cells express Trp10 transcripts as well as Trp10-antisense transcripts. S, no Trp10 expression is detectable in benign hyperplasia of the prostate (BPH). O and T, show expression of Trp10 transcripts (O) and Trp10-antisense transcripts (T) in a metastasis of a melanoma in human lung. Melanoma cancer cells express both Trp10 transcripts and Trp10-antisense transcripts.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to an isolated nucleic acid molecule encoding the human prostate carcinoma associated protein Trp8a, Trp8b, Trp9, Trp10a or Trp10b or a protein exhibiting biological properties of Trp8a, Trp8b, Trp9, Trp10a or Trp10b and being selected from the group consisting of

(a) a nucleic acid molecule encoding a protein that comprises the amino acid sequence depicted in Figure 7, 8A, 9,10 or 11;

(b) a nucleic acid molecule comprising the nucleotide sequence depicted in Figure 7, 8A, 9,10, or 11;

(c) a nucleic acid molecule included in DSMZ Deposit no. DSM 13579 (deposit date: 28 June 2000), DSM 13580 (deposit date: 28 June 2000), DSM 13584 (deposit date: 5 July 2000), DSM 13581 (deposit date: 28 June 2000) or DSM ....(deposit date:....);

(d) a nucleic acid molecule with hybridizes to a nucleic acid molecule specified in (a) to (c)

(e) a nucleic acid molecule the nucleic acid sequence of which deviates from the nucleic sequences specified in (a) to (d) due to the degeneration of the genetic code; and

(f) a nucleic acid molecule, which represents a fragment, derivative or allelic variation of a nucleic acid sequence specified in (a) to (e).

As used herein, a protein exhibiting biological properties of Trp8a, Trp8b, Trp9,Trp10a or Trp10b is understood to be a protein having at least one of the activities as illustrated in the Examples, below.

As used herein, the term „isolated nucleic acid molecule„ includes nucleic acid molecules substantially free of other nucleic acids, proteins, lipids, carbohydrates or other materials with which it is naturally associated.

In a first embodiment, the invention provides an isolated nucleic acid molecule encoding the human prostate carcinoma associated protein Trp8a, Trp8b, Trp9,Trp10a or Trp10b comprising the amino acid sequence depicted in Figure 7, 8A, 9,10 or 11. The present invention also provides a nucleic acid molecule comprising the nucleotide sequence depicted in Figure 7, 8A, 9,10 or 11.

The present invention provides not only the generated nucleotide sequence identified in Figure 7, 8A, 9,10 or 11, respectively and the predicted translated amino acid sequence, respectively, but also plasmid DNA containing a Trp8a cDNA deposited with the DSMZ, under DSM 13579, a Trp8b cDNA deposited with the DSMZ, under DSM 13580, a Trp9 cDNA deposited with the DSMZ, under DSM 13584, a Trp10a cDNA deposited with the DSMZ, under DSM 13581, and a Trp10b cDNA deposited with the DSMZ, under DSM...., respectively. The nucleotide sequence of each deposited Trp-clone can readily be determined by sequencing the deposited clone in accordance with known methods. The predicted amino acid sequence can then be verified from such deposits. Moreover, the amino acid sequence of the protein encoded by each deposited clone can also be directly determined by peptide sequencing or by expressing the protein in a suitable host cell containing the deposited Trp-encoding DNA, collecting the protein, and determining its sequence.

The nucleic acid molecules of the invention can be both DNA and RNA molecules. Suitable DNA molecules are, for example, genomic or cDNA molecules. It is understood that all

nucleic acid molecules encoding all or a portion of Trp8a, Trp8b, Trp9,Trp10a or Trp10b are also included, as long as they encode a polypeptide with biological activity. The nucleic acid molecules of the invention an be isolated from natural sources or can be synthesized according to know methods.

The present invention also provides nucleic acid molecules which hybridize to the above nucleic acid molecules. As used herein, the term „hybridize„ has the meaning of hybridization under conventional hybridization conditions, preferably under stringent conditions as described, for example, in Sambrook et al., Molecular Cloning, A Laboratory Manual 2nd edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. Also contemplated are nucleic acid molecules that hybridize to the Trp nucleic acid molecules at lower stringency. hybridization conditions. Changes in the stringency of hybridization and signal detection are primarily accomplished through the manipulation of formamide concentration (lower percentages of formamide result in lowered stringency), salt conditions, or temperature. For example, lower stringency conditions include an overnight incubation at 37°Cin a solution comprising 6X SSPE (20X SSPE = 3M NaCl; 9.2M NaH_2PO_4; 0.02M EDTA, pH7.4), 0.5% SDS, 30% formamide, 100 µg/ml salmon sperm blocking DNA, following by washes at 50°C with 1 X SSPE, 0.1% SDS. In addition, to achieve even lower stringency, washes performed following stringent hybridization can be done at higher salt concentrations (e.g. 5X SSC). Variations in the above conditions may be accomplished through the inclusion and/or substitution of alternate blocking reagents used to suppress background in hybridization experiments. The inclusion of specific blocking reagents may require modification of the hybridization conditions described above, due to problems with compatibility.

Nucleic acid molecules that hybridize to the molecules of the invention can be isolated, e.g., from genomic or cDNA libraries that were produced from human cell lines or tissues. In order to identify and isolate such nucleic acid molecules the molecules of the invention or parts of these molecules or the reverse complements of these molecules can be used, for example by means of hybridization according to conventional methods (see, e.g., Sambrook et al., supra). As a hybridization probe nucleic acid molecules can be used, for example, that have exactly or basically the nucleotide sequence depicted in Figure 7, 8A, 9,10 or 11, respectively, or parts of these sequences. The fragments used as hybridization probe can be synthetic

fragments that were produced by means of conventional synthetic methods and the sequence of which basically corresponds to the sequence of a nucleic acid molecule of the invention.

The nucleic acid molecules of the present invention also include molecules with sequences that are degenerate as a result of the genetic code.

In a further embodiment, the present invention provides nucleic acid molecules which comprise fragments, derivatives and allelic variants of the nucleic acid molecules described above encoding a protein of the invention. „Fragments„ are understood to be parts of the nucleic acid molecules that are long enough to encode one of the described proteins. These fragments comprise nucleic acid molecules specifically hybridizing to transcripts of the nucleic acid molecules of the invention. These nucleic acid molecules can be used, for example, as probes or primers in the diagnostic assay and/or kit described below and, preferably, are oligonucleotides having a length of at least 10, in particular of at least 15 and particularly preferred of at least 50 nucleotides. The nucleic acid molecules and oligonucleotides of the invention can also be used, for example, as primers for a PCR reaction. Examples of particular useful probes (primers) are shown in Tables 1 and 2.

Table 1

Trp8 probes used for in situ hybridization:

Probes (antisense)

1.) 5' TCCGCTGCCGGTTGAGATCTTGCC 3'

2.) 5' CTTGCTCCATAGGCAGAGAATTAG 3'

3.) 5' ATCCTCAGAGCCCCGGGTGTGGAA3'

Controls (sense)

1.) 5' GGCAAGATCTCAACCGGCAGCGGA 3'

2.) 5' CTAATTCTCTGCCTATGGAGCAAG 3'

3.) 5' TTCCACACCCGGGGCTCTGAGGAT 3'

Tabelle 2

Trp10 probes used for the in situ hybridizations shown in Figure 14:

Probes (antisense)

1.) 5' GCTTCCACCCCAAGCTTCACAGGAATAGA 3' (Figure 14 A, 14B)

2.) 5' GGCGATGAAATGCTGGTCTGTGGC 3' (Figure 14C, 14D, 14N, 14S, 14O)

3.) 5' ATCTTCCAGTTCTTGGTGTCTCGG 3' (Figure 14E, 14K)

4.) 5' GCTGCAGTACTCCTGCACCAGGAA 3' (Figure 14L, 14M)

Probes (sense)

1.) 5' TCTATTCCTGTGAAGCTTGGGGTGGAAGC 3' (Figure 14F, 14G)

2.) 5' GCCACAGACCAGCATTTCATCGCC 3' (Figure 14H, 14I, 14T)

3.) 5' CCGAGACACCAAGAACTGGAAGAT 3' (Figure 14J, 14P)

4.) 5' TTCCTGGTGCAGGAGTACTGCAGC 3' (Figure 14Q, 14R)

The term „derivative„ in this context means that the sequences of these molecules differ from the sequences of the nucleic acid molecules described above at one or several positions but have a high level of homology to these sequences. Homology hereby means a sequence identity of at least 40%, in particular an identity of at least 60%, preferably of more than 80% and particularly preferred of more than 90%. These proteins encoded by the nucleic acid molecules have a sequence identity to the amino acid sequence depicted in Figure 7, 8A, 9, 10 and 11, respectively, of at least 80%, preferably of 85% and particularly preferred of more than 90%, 97% and 99%. The deviations to the above-described nucleic acid molecules may have been produced by deletion, substitution, insertion or recombination. The definition of the derivatives also includes splice variants, e.g. the splice variants shown in Figures 8B to 8E and 9B.

The nucleic acid molecules that are homologous to the above-described molecules and that represent derivatives of these molecules usually are variations of these molecules that represent modifications having the same biological function. They can be naturally occurring variations, for example sequences from other organisms, or mutations that can either occur naturally or that have been introduced by specific mutagenesis. Furthermore the variations can be synthetically produced sequences. The allelic variants can be either naturally occurring variants or synthetically produced variants or variants produced by recombinant DNA processes.

Generally, by means of conventional molecular biological processes it is possible (see, e.g., Sambrook et al., supra) to introduce different mutations into the nucleic acid molecules of the invention. As a result Trp proteins or Trp related proteins with possibly modified biological properties are synthesized. One possibility is the production of deletion mutants in which nucleic acid molecules are produced by continuous deletions from the 5'- or 3'-terminal of the coding DNA sequence and that lead to the synthesis of proteins that are shortened accordingly. Another possibility is the introduction of single-point mutation at positions where a modification of the amino aid sequence influences, e.g., the ion channel properties or the regulations of the trp-ion channel. By this method muteins can be produced, for example, that possess a modified ion conducting pore, a modified K_m-value or that are no longer subject to the regulation mechanisms that normally exist in the cell, e.g. with regard to allosteric regulation or covalent modification. Such muteins might also be valuable as therapeutically useful antagonists of Trp8a, Trp8b, Trp9,Trp10a or Trp10b, respectively.

For the manipulation in prokaryotic cells by means of genetic engineering the nucleic acid molecules of the invention or parts of these molecules can be introduced into plasmids allowing a mutagenesis or a modification of a sequence by recombination of DNA sequences. By means of conventional methods (cf. Sambrook et al., supra) bases can be exchanged and natural or synthetic sequences can be added. In order to link the DNA fragments with each other adapters or linkers can be added to the fragments. Furthermore, manipulations can be performed that provide suitable cleavage sites or that remove superfluous DNA or cleavage sites. If insertions, deletions or substitutions are possible, in vitro mutagenesis, primer repair, restriction or ligation can be performed. As analysis method usually sequence analysis, restriction analysis and other biochemical or molecular biological methods are used.

The proteins encoded by the various variants of the nucleic acid molecules of the invention show certain common characteristics, such as ion channel activity, molecular weight, immunological reactivity or conformation or physical properties like the electrophoretical mobilty, chromatographic behavior, sedimentation coefficients, solubility, spectroscopic properties, stability; pH optimum, temperature optimum.

The invention furthermore relates to vectors containing the nucleic acid molecules of the invention. Preferably, they are plasmids, cosmids, viruses, bacteriophages and other vectors

usually used in the field of genetic engineering. Vectors suitable for use in the present invention include, but are not limited to the T7-based expression vector for expression in mammalian cells and baculovirus-derived vectors for expression in insect cells. Preferably, the nucleic acid molecule of the invention is operatively linked to the regulatory elements in the recombinant vector of the invention that guarantee the transcription and synthesis of an RNA in prokryotic and/or eukaryotic cells that can be translated. The nucleotide sequence to be transcribed can be operably linked to a promoter like a T7, metallothionein I or polyhedrin promoter.

In a further embodiment, the present invention relates to recombinant host cells transiently or stable containing the nucleic acid molecules or vectors or the invention. A host cell is understood to be an organism that is capable to take up *in vitro* recombinant DNA and, if the case may be, to synthesize the proteins encoded by the nucleic acid molecules of the invention. Preferably, these cells are prokaryotic or eukaryotic cells, for example mammalian cells, bacterial cells, insect cells or yeast cells. The host cells of the invention are preferably characterized by the fact that the introduced nucleic acid molecule of the invention either is heterologous with regard to the transformed cell, i.e. that it does not naturally occur in these cells, or is localized at a place in the genome different from that of the corresponding naturally occurring sequence.

A further embodiment of the invention relates to isolated proteins exhibiting biological properties of the human prostate carcinoma associated protein Trp8a, Trp8b, Trp9,Trp10a or Trp10b and being encoded by the nucleic acid molecules of the invention, as well as to methods for their production, whereby, e.g., a host cell of the invention is cultivated under conditions allowing the synthesis of the protein and the protein is subsequently isolated from the cultivated cells and/or the culture medium. Isolation and purification of the recombinantly produced proteins may be carried out by conventional means including preparative chromatography and affinity and immunological separations involving affinity with an anti-Trp8a-, anti-Trp8b-, anti-Trp9-,anti-Trp10a- or anti-Trp10b-antibody, respectively.

As used herein, the term „isolated protein„ includes proteins substantially free of other proteins, nucleic acids, lipids, carbohydrates or other materials with which it is naturally associated. Such proteins however not only comprise recombinantly produced proteins but include isolated naturally occurring proteins, synthetically produced proteins, or proteins

produced by a combination of these methods. Means for preparing such proteins are well understood in the art. The Trp proteins are preferably in a substantially purified form. A recombinantly produced version of a human prostate carcinoma associated protein Trp8a, Trp8b, Trp9,Trp10a or Trp10b protein, including the secreted protein, can be substantially purified by the one-step method described in Smith and Johnson, Gene 67; 31-40 (1988).

In a further preferred embodiment, the present invention relates to an antisense RNA sequence characterised that it is complementary to an mRNA transcribed from a nucleic acid molecule of the present invention or a part thereof and can selectively bind to said mRNA, said sequence being capable of inhibiting the synthesis of the protein encoded by said nucleic acid molecules, and a ribozyme characterised in that it is complementary to an mRNA transcribed from a nucleic acid molecule of the present invention or a part thereof and can selectively bind to and cleave said mRNA, thus inhibiting the synthesis of the proteins encoded by said nucleic acid molecules. Riboyzmes which are composed of a single RNA chain are RNA enzymes, i.e. catalytic RNAs, which can intermolecularly cleave a target RNA, for example the mRNA transcribed from one of the Trp genes. It is now possible to construct ribozymes which are able to cleave the target RNA at a specific site by following the strategies described in the literature. (see, e.g., Tanner et al., in: Antisense Research and Applications, CRC Press Inc. (1993), 415-426). The two main requirements for such ribozymes are the catalytic domain and regions which are complementary to the target RNA and which allow them to bind to its substrate, which is a prerequisite for cleavage. Said complementary sequences, i.e., the antisense RNA or ribozyme, are useful for repression of Trp8a-, Trp8b, Trp9-,Trp10a- and Trp10b-expression, respectively, i.e. in the case of the treatment of a prostate cancer or endometrial cancer (carcinoma of the uterus). Preferably, the antisense RNA and ribozyme of the invention are complementary to the coding region. The person skilled in the art provided with the sequences of the nucleic acid molecules of the present invention will be in a position to produce and utilise the above described antisense RNAs or ribozymes. The region of the antisense RNA and ribozyme, respectively, which shows complementarity to the mRNA transcribed from the nucleic acid molecules of the present invention preferably has a length of at least 10, in particular of at least 15 and particularly preferred of at least 50 nucleotides.

In still a further embodiment, the present invention relates to inhibitors of Trp8a, Trp8b, Trp9, Trp10a and Trp10b, respectively, which fulfill a similar purpose as the antisense RNAs or

ribozymes mentioned above, i.e. reduction or elimination of biologically active Trp8a, Trp8b, Trp9, Trp10a or Trp10b molecules. Such inhibitors can be, for instance, structural analogues of the corresponding protein that act as antagonists. In addition, such inhibitors comprise molecules identified by the use of the recombinantly produced proteins, e.g. the recombinantly produces protein can be used to screen for and identify inhibitors, for example, by exploiting the capability of potential inhibitors to bind to the protein under appropriate conditions. The inhibitors can, for example, be identified by preparing a test mixture wherein the inhibitor candidate is incubated with Trp8a, Trp8b, Trp9, Trp10a or Trp10b, respectively, under appropriate conditions that allow Trp8a, Trp8b, Trp9, Trp10a or Trp10b to be in a native conformation. Such an in vitro test system can be established according to methods well known in the art. Inhibitors can be identified, for example, by first screening for either synthetic or naturally occurring molecules that bind to the recombinantly produced Trp protein and then, in a second step, by testing those selected molecules in cellular assays for inhibition of the Trp protein, as reflected by inhibition of at least one of the biological activities as described in the examples, below. Such screening for molecules that bind Trp8a, Trp8b, Trp9, Trp10a or Trp10b could easily performed on a large scale, e.g. by screening candidate molecules from libraries of synthetic and/or natural molecules. Such an inhibitor is, e.g., a synthetic organic chemical, a natural fermentation product, a substance extracted from a microorganism, plant or animal, or a peptide. Additional examples of inhibitors are specific antibodies, preferably monoclonal antibodies. Moreover, the nucleic sequences of the invention and the encoded proteins can be used to identify further factors involved in tumor development and progression. In this context it should be emphasized that the modulation of the calcium channel of a member of the trp family can result in the stimulation of the immune response of T lymphocytes leading to proliferation of the T lymphocytes. The proteins of the invention can, e.g., be used to identify further (unrelated) proteins which are associated with the tumor using screening methods based on protein/protein interactions, e.g. the two-hybrid-system Fields, S. and Song, O. (1989) Nature (340): 245-246.

The present invention also provides a method for diagnosing a prostate carcinoma which comprises contacting a target sample suspected to contain the protein Trp8a, Trp8b, Trp10a and/or Trp10b or the Trp8a, Trp8b, Trp10a and/or Trp10b encoding mRNA with a reagent which reacts with Trp8a, Trp8b, Trp10a and/or Trp10b or the Trp8a, Trp8b, Trp10a and/or Trp10b encoding mRNA and detecting Trp8a, Trp8b, Trp10a and/or Trp10b or Trp8a, Trp8b, Trp10a and/or Trp 10b encoding mRNA.

It has been found that carcinoma cells of placenta (chorion carcinoma), lung and prostate express Trp10 transcripts as well as Trp10 antisense transcripts and transcripts being in part complementary to Trp10 antisense transcripts. Accordingly, the present invention also provides a method for diagnosing a melanoma, chorion carcinoma, cancer of the lung and of the prostate in a tissue of a subject, comprising contacting a sample with a reagent which detects Trp10a and/or Trp10b antisense RNA.

When the target is mRNA (or antisense RNA), the reagent is typically a nucleic acid probe or a primer for PCR. The person skilled in the art is in a position to design suitable nucleic acids probes based on the information as regards the nucleotide sequence of Trp8a, Trp8b, Trp10a or Trp10b as depicted in figure 7, 8a, 10 and 11, respectively, or tables 1 and 2, above. When the target is the protein, the reagent is typically an antibody probe. The term „antibody", preferably, relates to antibodies which consist essentially of pooled monoclonal antibodies with different epitopic specifities, as well as distinct monoclonal antibody preparations. Monoclonal antibodies are made from an antigen containing fragments of the proteins of the invention by methods well known to those skilled in the art (see, e.g., Köhler et al., Nature 256 (1975), 495). As used herein, the term „antibody" (Ab) or „monoclonal antibody" (Mab) is meant to include intact molecules as well as antibody fragments (such as, for example, Fab and F(ab') 2 fragments) which are capable of specifically binding to protein. Fab and f(ab')2 fragments lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and may have less non-specific tissue binding than an intact antibody. (Wahl et al., J. Nucl. Med. 24: 316-325 (1983)). Thus, these fragments are preferred, as well as the products of a FAB or other immunoglobulin expression library. Moreover, antibodies of the present invention include chimerical, single chain, and humanized antibodies. The target cellular component, i.e. Trp8a, Trp8b, Trp10a and/or Trp10b or Trp8a, Trp8b, Trp10a and/or Trp10b encoding mRNA or Trp10a/b antisense transcripts, e.g., in biological fluids or tissues, may be detected directly in situ, e.g. by in situ hybridization (e.g., according to the examples, below) or it may be isolated from other cell components by common methods known to those skilled in the art before contacting with a probe. Detection methods include Northern blot analysis, RNase protection, in situ methods, e.g. in situ hybridization, in vitro amplification methods (PCR, LCR, QRNA replicase or RNA-transcription/amplification (TAS, 3SR), reverse dot blot disclosed in EP-B1 O 237 362)), immunoassays, Western blot and other detection assays that are known to those skilled in the art.

Products obtained by in vitro amplification can be detected according to established methods, e.g. by separating the products on agarose gels and by subsequent staining with ethidium bromide. Alternatively, the amplified products can be detected by using labeled primers for amplification or labeled dNTPs.

The probes can be detectable labeled, for example, with a radioisotope, a bioluminescent, compound, a chemiluminescent compound, a fluorescent compound, a metal chelate, or an enzyme.

Expression of Trp8a, Trp8b, Trp10a and Trp10b, respectively, in tissues can be studied with classical immunohistological methods (Jalkanen et al., J. Cell. Biol. 101 (1985), 976-985; Jalkanen et al., J. Cell. Biol. 105 (1987), 3087-3096; Sobol et al. Clin. Immunpathol. 24 (1982), 139-144; Sobol et al., Cancer 65 (1985), 2005-2010). Other antibody based methods useful for detecting protein gene expression include immunoassays, such as the enzyme linked immunosorbent assay (ELISA) and the radioimmunoassay (RIA). Suitable antibody assay labels are known in the art and include enzyme labels, such as, glucose oxidase, and radioisotopes, such as iodine (^{125}I, ^{121}I), carbon (^{14}C), sulfur (^{35}S), tritium (^{3}H), indium (^{112}In), and technetium rhodamine, and biotin. In addition to assaying Trp8a, Trp8b, Trp 10a or Trp10b levels in a biological sample, the protein can also be detected in vivo by imaging. Antibody labels or markers for in vivo imaging of protein include those detectable by X-radiography, NMR or ESR. For X-radiography, suitable labels include radioisotopes such as barium or cesium, which emit detectable radiation but are not overtly harmful to the subject. Suitable markers for NMR and ESR include those with a detectable characteristic spin, such as deuterium, which may be incorporated into the antibody by labeling of nutrients for the relevant hybridoma. A protein-specific antibody or antibody fragment which has been labeled with an appropriate detectable imaging moiety, such as a radioisotope (for example, ^{131}I, ^{112}In, ^{99}mTc), a radio-opaque substance, or a material detectable by nuclear magnetic resonance, is introduced (for example, parenterally, subcutaneously, or intraperitoneally) into the mammal. It will be understood in the art that the size of the subject and the imaging system used will determine the quantity of imaging moiety needed to produce diagnostic images. In the case of a radioisotope moiety, for a human subject, the quantity of radioactivity injected will normally range from about 5 to 20 millicuries of ^{99}mTc. The labeled antibody or antibody fragment will then preferentially accumulate at he location of cells which contain the specific protein. In

vivo tumor imaging is described in S.W. Burchiel et al., „Immunopharmacokinetics of Radiolabeled Antibodies and Their Fragments". (Chapter 13 in Tumor Imaging: The Radiochemical Detection of Cancer, S.W. Burchiel and B.A. Rhodes, eds., Masson Publishing Inc. (1982)).

The marker Trp8a and Trp8b is also useful for prognosis, for monitoring the progression of the tumor and the diagnostic evaluation of the degree of malignancy of a prostate tumor (grading and staging), e.g. by using in situ hybridization: In a primary carcinoma Trp8 is expressed in about 2 to 10% of carcinoma cells, in a rezidive carcinoma in about 10 to 60% of cells and in metastases in about 60 to 90% of cells.

The present invention also relates to a method for diagnosing endometrial cancer (cancer of the uterus) which comprises contacting a target sample suspected to contain the protein Trp8a and/or Trp8b or the Trp8a and/or Trp8b encoding mRNA with a reagent which reacts with Trp8a and/or Trp8b or the encoding mRNA and detecting Trp8a and/or Trp8b encoding mRNA. As regards particular embodiments of this method reference is made to the particular embodiments of the method of diagnosing a prostate cancer outlined above.

For evaluating whether the concentration of Trp8a, Trp8b, Trp10a or Trp10b or the concentration of Trp8a, Trp8b, Trp10a or Trp10b encoding mRNA is normal or increased, thus indicative for the presence of a malignant tumor, the measured concentration is compared with the concentration in a normal tissue, preferably by using the ratio of Trp8a:Trp9, Trp8b:Trp9 or Trp10(a or b)/Trp9 for quantification.

Since the prostate carcinoma forms its own basement membrane when growing invasively, it can be concluded that only cells expressing Trp8 and Trp10 are involved in this phenomenon. Thus, it can be concluded that by inhibiting the expression and/or activity of these proteins an effective therapy of cancers like PCA is provided.

Thus, the present invention also relates to a pharmaceutical composition containing a reagent which decreases or inhibits Trp8a, Trp8b, Trp10a and/or Trp10b expression or the activity of Trp8a, Trp8b, Trp10a and/or Trp10b, and a method for preventing, treating, or ameliorating a prostate tumor, endometrial cancer (uterine carcinoma) tumor, a chorion carcinoma, cancer of the lung or melanoma,which comprises administering to a mammalian subject a

therapeutically effective amount of a reagent which decreases or inhibits Trp8a, Trp8b, Trp10a and/or Trp10b expression or the activity of Trp8a, Trp8b, Trp10a and/or Trp10b. Examples of such reagents are the above described antisense RNAs, ribozymes or inhibitors, e.g. specific antibodies. Furthermore, peptides, which inhibit or modulate the biological function of Trp8a, Trp8b, Trp9, Trp10a and/or Trp10b may be useful as therapeutical reagents. For example, these peptides can be obtained by screening combina torial phage display libraries (Cosmix, Braunschweig, Germany) as described by Rottgen, P. and Collins, J. (Gene (1995) 164 (2): 243-250). Furthermore, antigenic epitopes of the Trp8 and Trp10 proteins can be identified by the expression of recombinant Trp8 and Trp10 epitope libraries in E. coli (Marquart, A. & Flockerzi, V., FEBS Lett. 407 (1997), 137-140; Trost, C., et al., FEBS Lett. 451 (1999) 257-263 and the consecutive screening of these libraries with serum of patients with cancer of the prostate or of the endometrium. Those Trp8 and Trp10 epitopes which are immunogenic and which lead to the formation of antibodies in the serum of the patients can be then be used as Trp8 or Trp10 derived peptide vaccines for immune inventions against cancer cells which express Trp8 or Trp10. Alternatively to the E. coli expression system, Trp8 or Trp10 or epitopes of Trp8 and Trp10 can be expressed in mammalian cell lines such as human embryonic kidney (Hek 293) cells (American Type Culture Collection, ATCC CRL 1573).

Finally, compounds useful for therapy of the above described diseases comprise compounds which act as antagonists or agonists on the ion channels Trp8, Trp9 and Trp10. It could be shown that Trp8 is a highly calcium selective ion channel which in the presence of monovalent (namely sodium) and divalent ions (namely calcium) is only permeable for calcium ions (see Example 4, below, and Figures 3A, C, E). Under physiological conditions, Trp8 is a calcium selective channel exhibiting large inward currents. This very large conductance of Trp8 channels (as wells as Trp9 and Trp10a/b channels) is useful to establish systems for screening pharmacological compounds interacting with Trp-channels including high throughput screening systems. Useful high throughput screening systems are well known to the person skilled in the art and include, e.g., the use of cell lines stably or transiently transfected with DNA sequences encoding Trp8, Trp9 and Trp10 channels in assays to detect calcium signaling in biological systems. Such systems include assays based on Ca-sensitive dyes such as aequorin, apoaequorin, Fura-2, Fluo-3 and Indo-1.

Accordingly, the present invention also relates to a method for identifying compounds which act as agonists or antagonists on the ion channels Trp8, Trp9 and/or Trp10, said method comprising contacting a test compound with the ion channel Trp8, Trp9 and/or Trp10, preferably by using a system based on cells stably or transiently transfected with DNA sequences encoding Trp8, Trp9 and/or Trp10, and determining whether said test compound affects the calcium uptake.

For administration the above described reagents are preferably combined with suitable pharmaceutical carriers. Examples of suitable pharmaceutical carriers are well known in the art and include phosphate buffered saline solutions, water, emulsions, such as oil/water emulsions, various types of wetting agents, sterile solutions etc. Such carriers can be formulated by conventional methods and can be administered to the subject at a suitable dose. Administration of the suitable compositions may be effected by different ways, e.g. by intravenous, intraperetoneal, subcutaneous, intramuscular, topical or intradermal administration. The route of administration, of course, depends on the nature of the tumor and the kind of compound contained in the pharmaceutical composition. The dosage regimen will be determined by the attending physician and other clinical factors. As is well known in the medical arts, dosages for any one patient depends on many factors, including the patient's size, body surface area, age, sex, the particular compound to be administered, time and route of administration, the kind and stage of the tumor, general health and other drugs being administered concurrently.

The delivery of the antisense RNAs or ribozymes of the invention can be achieved by direct application or, preferably, by using a recombinant expression vector such as a chimeric virus containing these compounds or a colloidal dispersion system. By delivering these nucleic acids to the desired target, the intracellular expression of Trp8a, Trp8b, Trp10a and/or Trp10b and, thus, the level of Trp8a, Trp8b, Trp10a and/or Trp10b can be decreased resulting in the inhibition of the negative effects of Trp8a, Trp8b, Trp10a and/or Trp10b, e.g. as regards the metastasis formation of PCA.

Direct application to the target site can be performed, e.g., by ballistic delivery, as a colloidal dispersion system or by catheter to a site in artery. The colloidal dispersion systems which can be used for delivery of the above nucleic, acids include macromolecule complexes, nanocapsules, microspheres, beads and lipid-based systems including oil-in-water emulsions

(mixed), micelles, liposomes and lipoplexes, The preferred colloidal system is a liposome. The composition of the liposome is usually a combination of phospholipids and steroids, especially cholesterol. The skilled person is in a position to select such liposomes which are suitable for the delivery of the desired nucleic acid molecule. Organ-specific or cell-specific liposomes can be used in order to achieve delivery only to the desired tumor. The targeting of liposomes can be carried out by the person skilled in the art by applying commonly known methods. This targeting includes passive targeting (utilizing the natural tendency of the liposomes to distribute to cells of the RES in organs which contain sinusoidal capillaries) or active targeting (for example by coupling the liposome to a specific ligand, e.g., an antibody, a receptor, sugar, glycolipid, protein etc., by well known methods). In the present invention monoclonal antibodies are preferably used to target liposomes to specific tumors via specific cell-surface ligands.

Preferred recombinant vectors useful for gene therapy are viral vectors, e.g. adenovirus, herpes virus, vaccinia, or, more preferably, an RNA virus such as a Retrovirus. Even more preferably, the retroviral vector is a derivative of a murine or avian retrovirus. Examples of such retroviral vectors which can be used in the present invention are: Moloney murine leukemia virus (MoMuLV), Harvey murine sarcoma virus (HaMuSV), murine mammary tumor virus (MuMTV) and Rous sarcoma virus (RSV). Most preferably, a non-human primate retroviral vector is employed, such as the gibbon ape leukemia virus (GaLV), providing a broader host range compared to murine vectors. Since recombinant retroviruses are defective, assistance is required in order to produce infectious particles. Such assistance can be provided, e.g., by using helper cell lines that contain plasmids encoding all of the structural genes of the retrovirus under the control of regulatory sequences within the LTR. Suitable helper cell lines are well known to those skilled in the art. Said vectors can additionally contain a gene encoding a selectable marker so that the transduced cells can be identified. Moreover, the retroviral vectors can be modified in such a way that they become target specific. This can be achieved, e.g., by inserting a polynucleotide encoding a sugar, a glycolipid, or a protein, preferably an antibody. Those skilled in the art know additional methods for generating target specific vectors. Further suitable vectors and methods for in vitro- or in vivo-gene therapy are described in the literature and are known to the persons skilled in the art; see, e.g., WO 94/29469 or WO 97/00957.

In order to achieve expression only in the target organ, i.e. tumor to be treated, the nucleic acids encoding, e.g. an antisense RNA or ribozyme can also be operably linked to a tissue specific promoter and used for gene therapy. Such promoters are well known to those skilled in the art (see e.g. Zimmermann et al., (1994) Neuron 12, 11-24; Vidal et al.; (1990) EMBO J. 9, 833-840; Mayford et al., (1995), Cell 81, 891-904; Pinkert et al., (1987) Genes & Dev. 1, 268-76).

For use in the diagnostic research discussed above, kits are also provided by the present invention. Such kits are useful for the detection of a target cellular component, which is Trp8a, Trp8b, Trp10a and/or Trp10b or, alternatively, Trp8a, Trp8b, Trp10a and/or Trp10b encoding mRNA or Trp10a/b antisense transcripts, wherein the presence or an increased concentration of Trp8a, Trp8b, Trp10a and/or Trp10b or, alternatively, Trp8a, Trp8b, Trp10a and/or Trp10b encoding mRNA or Trp10a/b antisense transcripts is indicative for a prostate tumor, endometrial cancer, melanoma, chorion carcinoma or cancer of the lung, said kit comprising a probe for detection of Trp8a, Trp8b, Trp9, Trp10a and/or Trp10b or, alternatively, Trp8a, Trp8b, Trp9, Trp10a and/or Trp10b encoding mRNA or Trp10a/b antisense transcripts. The probe can be detectably labeled. Such probe may be a specific antibody or specific oligonucleotide. In a preferred embodiment, said kit contains an anti-Trp8a-, anti-Trp8b-, anti-Trp9-, anti-Trp10a-and/or anti-Trp10b-antibody and allows said diagnosis, e.g., by ELISA and contains the antibody bound to a solid support, for example, a polystyrene microtiter dish or nitrocellulose paper, using techniques known in the art. Alternatively, said kits are based on a RIA and contain said antibody marked with a radioactive isotope. In a preferred embodiment of the kit of the invention the antibody is labeled with enzymes, fluorescent compounds, luminescent compounds, ferromagnetic probes or radioactive compounds. The kit of the invention may comprise one or more containers filled with, for example, one or more probes of the invention. Associated with container (s) of the kit can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, us or sale for human administration.

EXAMPLES

The following Examples are intended to illustrate, but not to limit the invention. While such Examples are typical of those that might be used, other methods known to those skilled in the art may alternatively be utilized.

Example 1: Materials and Methods

(A) Isolation of cDNA clones and Northern blot analysis

Total RNA was isolated from human placenta an prostate using standard techniques. Isolation of mRNA was performed with poly $(A)^+$RNA - spin columns (New England Biolabs, Beverly, USA) according to the instructions of the manufacturer. Poly (a) $^+$RNA was reverse transcribed using the cDNA choice system (Gibco-BRL, Rockville, USA) and subcloned in λ-Zap phages (Stratagene, La Jolla, USA). An human expressed sequence tag (GenBank accession number 1404042) was used to screen an oligo d(T) primed human placenta cDNA library. Several cDNA clones were identified and isolated. Additional cDNA clones were isolated from two specifically primed cDNA libraries using primers 5'-gca tag gaa ggg aca ggt gg-3' and 5'-gag agt cga ggt cag tgg tcc-3'.

cDNA clones were sequenced using a thermocycler (PE Applied Biosystems, USA) and Thermo Sequenase (Amersham Pharmacia Biotech Europe, Freiburg, Germany). DNA sequences were analyzed with an automated sequencer (Licor, Linccoln, USA).

For Northern blot analysis 5 µg human poly $(A)^+$ RNA from human placenta or prostate were separated by electrophoresis on 0.8 % agarose gels. Poly $(A)^+$ RNA was transferred to Hybond N nylon membranes (Amersham Pharmacia Biotech Europe, Freiburg, Germany). The membranes were hyridized in the presence of 50 % formamide at 42^0C over night. DNA probes were labelled using [α^{32}P]dCTP and the „ready prime„ labelling kit (Amersham Pharmacia Biotech Europe, Freiburg, Germany). Commercial Northern blots were hybridized according to the distributors instructions (Clontech, Paolo Alto, USA).

(B) Construction of expression plasmids and transfection of HEK 293 cells

Lipofections were carried out with the recombinant dicistronic eucaryotic expression plasmid pdiTRP8 containing the cDNA of Trp8b under the control of the chicken ß-actin promotor followed by an internal ribosome entry side (IRES) and the cDNA of the green fluorescent protein (GFP). To obtain pdiTRP8 carrying the entire protein coding regions of TRP8b and

the GFP (Prasher, D.C. et al. (1992), Gene 111, 229-233), the 5`and 3´-untranslated sequences of the TRP8b cDNA were removed, the consensus sequence for initiation of translation in vertebrates (Kozak, M. (1987) Nucleic Acids Research15, 8125-8148) was introduced immediately 5`of the translation initiation codon and the resulting cDNA was subcloned into the pCAGGS vector (Niwa, H., Yamamura, K. and Miyazaki, J (1991), Gene 8, 193-199) downstream of the chicken β-actin promotor. The IRES derived from encephalmyocarditis virus (Kim, D.G., Kang, H.M., Jang, S.K. and Shin H.S. (1992) Mol.Cell.Biol. 12, 3636-3643) followed by the GFP cDNA containing a Ser65Thr mutation (Heim, R., Cubitt, A.B., Tsien, R.Y. (1995) Nature 373, 663-664) was then cloned 3` to the TRP8b cDNA. The IRES sequence allows the simultaneous translation of TRP8b and GFP from one transcript. Thus, transfected cells can be detected unequivocally by the development of green fluorescence.

For monitoring of the intracellular Ca^{2+} concentration human embryonic kidney (HEK 293) cells were cotransfected with the pcDNA3-TRP8b vector and the pcDNA3-GFPvector in a molar ratio of 4 : 1 in the presence of lipofectamine (Quiagen, Hilden, Germany). To obtain pcDNA3-TRP8b the entire protein coding region of TRP8b including the consensus sequence for initiation of translation in vertebrates (Kozak, M. (1987) Nucleic Acids Research15, 8125-8148) was subcloned into the pcDNA3 vector (Invitrogen, Groningen, Netherlands). Calcium monitoring and patch clamp experiments were carried out two days and one day after transfection, respectively.

(C) Chromosomal localization of the Trp8 gene

The chromosomal localization of the human TRP8 gene was performed using NIGMS somatic hybrid mapping panel No.2 (Coriell Institute, Camden, NJ, USA) previously described (Drwinga, H.L., Toji, L.H., Kim, C.H., Greene, A.E., Mulivor, R.A. (1993) Genomics 16, 311-314; Dubois, B.L. and Naylor, S.L. (1993) Genomics 16, 315-319).

(D) In Vitro Translation, glutathione - sepharose and calmodulin agarose binding assay

N– and C-terminal Trp8-fragments were subcloned into the pGEX-4T2 vector (Amersham Pharmacia Europe, Freiburg, Germany) resulting in glutathione-S-transferase (GST)-Trp8 fusion constructs (Fig. 4). The GST-TRP8-fusion proteins were expressed in E. coli BL 21 cells and purified using glutathione - sepharose beads (Amersham Pharmacia Biotech Europe, Freiburg, Germany).

In vitro translation of human Trp8 cDNA and Xenopus laevis calmodulin cDNA (Davis, T.N. and Thorner, J. Proc.Natl.Acad.Sci. USA 86, 7909-7913.) was performed in the presence of ^{35}S-methionine using the TNT coupled transcription/translation kit (Promega, Madison, USA). Translation products were purified by gel fliltration (Sephadex G50, Amersham Pharmacia Biotech Europe, Freiburg, Germany) and equal amounts of ^{35}S labeled probes were incubated for 2 h with glutathione beads bound to GST - Trp8 or calmodulin - agarose (Calbiochem) in 50 mM Tris-HCl, pH 7.4, 0.1 % Triton X-100, 150 mM NaCl in the presence of 1 mM Ca^{2+} or 2 mM EGTA. After three washes, bound proteins were eluted with SDS sample buffer, fractionated by SDS-PAGE and ^{35}S labeled proteins were detected using a Phosphor Imager (Fujifilm, Tokyo, Japan).

(E) Calcium measurements

The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was determined by dual wavelength fura-2 fluorescence ratio measurements (Tsien, R.Y. (1988) Trends Neurosci. 11, 419-424) using a digital imaging system (T.I.L.L. Photonics, Planegg, Germany). HEK cells were grown in minimal essential medium in the presence of 10 % fetal calf serum and cotransfected with the pcDNA3-TRP8b vector and the pCDNA3-GFPvector as described above (B). Transfected cells were detected by development of green fluorescence. The cells were loaded with 4μM fura-2/AM (Molecular Probes, Oregon, USA) for one hour. After loading the cells were rinsed 3 times with buffer B1 (10 mM Hepes, 115 mM NaCl, 2 mM $MgCl_2$, 5mM KCl, pH 7.4) and the $[Ca^{2+}]_i$ was calculated from the fluorescence ratios obtained at 340 and 380 nm excitation wavelengths as described (Garcia, D.E., Cavalié, A. and Lux, H.D. (1994) J. Neurosci 14, 545-553).

(F) Electrophysiological recordings

HEK cells were transfected with the eucaryotic expression plasmid pdiTRP8 described in (B) and electrophysiolocigal recordings were carried out one day after transfection. Single cells were voltage clamped in the whole cell mode of the patch clamp technique as described (Hamill, O.P., Marty, A., Neher, E., Sakmann, B. and Sigworth, F.J. (1981) Pflügers Arch. 391, 85-100; Philipp, S., Cavalié, A., Freichel, M., Wissenbach, U., Zimmer, S., Trost, C., Marquart, A., Murakami, M. and Flockerzi, V. (1996) EMBO J. 6166-6171). The pipette solution contained contained (mM): 140 aspartic acid, 10 EGTA, 10 NaCl, 1 MgCl2, 10 Hepes (pH 7.2 with CsOH) or 125 CsCl, 10 EGTA, 4 $CaCl_2$ 10 Hepes (pH 7,2 with CsOH). The bath solution contained (mM): 100 NaCl, 10 CsCl, 2 $MgCl_2$, 50 mannitol, 10 glucose, 20

Hepes (pH 7,4 with CsOH) and 2 $CaCl_2$, or no added $CaCl_2$ (-Ca^{2+} solution). Divalent free bath solution contained (mM): 110 N-methyl-D-glucamine (NMDG). Whole cell currents were recorded during 100 msec voltage ramps from –100 to +100 mV at varying holding potentials.

(G) In Situ Hybridization

In situ hybridizations were carried out using formalin fixed tissue slices of 6 - 8 µM thickness. The slices were hydrated and incubated in the presence of PBS buffer including 10 µg / ml proteinase K (Roche Diagnostics, Mannheim, Germany) for 0.5 h. The slices were hybridized at 37°C using biotinylated deoxy-oligonucleotides (0.5 pmol / µl) in the presence of 33 % formamide for 12 h. Furthermore the slices were several times rinsed with 2 x SSC and incubated at 25°C for 0.5 h with avidin / biotinylated horse raddish peroxidase complex (ABC, DAKO, Santa Barbara, USA). After several washes with PBS buffer the slices were incubated in the presence of biotinylated tyramid and peroxide (0.15 % w/v) for 10 min, rinsed with PBS buffer and additionally incubated with ABC complex for 0.5 h. The slices were washed with PBS buffer and incubated in the presence of DAB solution (diaminobenzidine (50µg / ml), 50 mM Tris/EDTA buffer pH 8.4, 0.15 % H_2O_2 in N,N - dimethyl-formamide; Merck, Darmstadt, Germany), The detection was stopped after 4 minutes by incubating the slides in water. Tyramid was biotinylated by incubating NHS-LC Biotin (sulfosuccinimidyl-6-(biotinimid)-hexanoat), 2.5 mg / ml; Pierce, Rockford, USA) and tyramin-HCl (0.75 mg / ml, Sigma) in 25 mM borate buffer pH 8.5 for 12 h. The tyramid solution was diluted 1 - 5 : 1000 in PBS buffer.

(H) GenBank accession numbers: TRP8a, Aj243500; TRP8b Aj243501

Example 2: Expression of TRP8 transcripts

In search of proteins distantly related to the TRP family of ion channels, an human expressed sequence tag (EST, GenBank accession number 1404042) was identified in the GenBank database using BLAST programms (at the National Center for Biotechnology Information (NCBI); Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J.J. (1990) Mol. Biol. 5, 403-410) being slightly homologous to the VR1 gene. Several human placenta cDNA libraries were constructed and screeened with this EST DNA as probe. Several full length

cDNA clones were identified and isolated. The full length cDNA clones encoded two putative proteins differing in three amino acids and were termed Trp8a and Trp8b (Fig. 1c, 2a, 7 and 8A). This finding was reproduced by isolating cDNA clones from two cDNA libraries constructed from two individual placentas. The derived protein sequence(s) comprises six transmembrane domains, a characteristic overall feature of trp channels and related proteins (Fig.: 1b). The sequence is closely related to the meanwhile published calcium uptake transport protein 1 (CaT1), isolated from rat intestine (Peng, J.B., Chen, X.Z., Berger, U.V., Vassilev, P.M., Tsukaguchi, H., Brown, E.M. and Hediger M.A.(1999) J Biol Chem. 6;274, 22739-22746) and to the epithelial calcium uptake channel (ECaC) isolated from rabbit kidney (Hoenderop, J.G., van der Kemp, A.W., Hartog, A., van de Graaf, S.F., van Os, C.H., Willems, P.H. and Bindels, R.J. (1999) J Biol Chem. 26;274, 8375-8378). Expression of Trp8a/b transcripts are detectable in human placenta, pancreas and prostate (Fig.: 5) and the size of the Northern signal (3.0 kb) corresponds with the size of the isolated full length cDNAs. In addition, a shorter transcript of 1.8 kb, probably a splice variant, is detectable in human testis. The Trp8 mRNA is not expressed in small intestine or colon (Fig.: 5) implicating that Trp8 is not the human ortholog of the rat CaT1 or rabbit ECaC proteins. To investigate whether there are other related sequences Trp8a/b derived primers (UW241, 5'-TAT GAG GGT TCA GAC TGC-3' and UW242, 5'-CAA AGT AGA TGA GGT TGC-3') were used to amplify a 105 bp fragment from human genomic DNA being 95% identical on the nucleotide level to the Trp8 sequence (data not shown). This indicates the existence of several similar sequences in humans at least at the genomic level.

Example 3: Two variants of the Trp8 protein (Trp8a and Trp8b) arise by polymorphism

Two variants of the Trp8 cDNA were isolated from human placenta (Fig.: 2A, 7 and 8A) which encoded two proteins which differ in three amino acids and were termed Trp8a and Trp8b. Trp8a/b specific primers were designed to amplify a DNA fragment of 458 bp of the Trp8 gene from genomic DNA isolated from human T-lymphocytes (primer pair: UW243, 5'-CAC CAT GTG CTG CAT CTA CC-3' and UW244, 5'-CAA TGA CAG TCA CCA GCT CC-3'). The amplification product contains a part of the sequence where the derived protein sequence of Trp8a comprises the amino acid valine and the Trp8b sequence methionine as well as a silent base pair exchange (g versus a) and an intron of 303bp (Fig.: 2.A, B). Both variants of the Trp8 genes (a,b) were amplified from genomic DNA in equal amounts indicating the existence of both variants in the human genome and therefore being not the

result of RNA editing (Fig.: 2B). The Trp8a gene can be distinguished from the Trp8b gene by cutting the genomic fragment of 458bp with the restriction enzyme Bsp1286I (Fig. 2B). Using human genomic DNA isolated from blood of twelve human subjects as template, the 458bp fragment was amplified and restricted with BSP1286I. In eleven of the tested subjects only the Trp8b gene is detectable, while one subject (7) contains Trp8a and Trp8b genes (Fig.: 2D). These implicates that the two Trp8 variants arise by polymorphism and do not represent individual genes. Using Trp8 specific primers and chromosomal DNA as template, the Trp8 locus is detectable on chromosome 7 (Fig.: 2C).

Example 4: Trp8b is a calcium permeable channel

The protein coding sequence of the Trp8b cDNA was subcloned into pcDNA3 vector (Invitrogen, Groningen, Netherlands) under the control of the cytomegalovirus promotor (CMV). Human embryonic kidney (HEK 293) cells were cotransfected with the Trp8b pcDNA3 construct (pcDNA3-Trp8b vector) and the pcDNA3-GFPvector encoding the green fluorescent protein (GFP) in 4:1 ratio. The Trp8b cDNA and the cDNA of the reporter, GFP, was transiently expressed in human embryonic kidney (HEK 293) cells. The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and changes of $[Ca^{2+}]_i$ were determined by dual wavelength fura-2 fluorescence ratio measurements (Fig.: 3F) in cotransfected cells which were identified by the green fluorescence of the reporter gene GFP.

Dual wavelength fura-2 fluorescence ratio measurement is a standard procedure (e.g. in: An introduction of Molecular Neurobiology (ed. Hall, Z.W.)Sinauer Associates, Sunderland, USA (1992)) using fura-2, which is a fluorescent Ca^{2+} sensitive dye and which was designed by R.Y.Tsien (e.g. Trends Neurosci. 11, 419-424 (1988) based upon the structure of EGTA. Its fluorescence emission spectrum is altered by binding to Ca^{2+} in the physiological concentration range. In the absence of Ca^{2+}, fura-2 fluoresces most strongly at an excitation wavelength of 385 nm; when it binds Ca^{2+}, the most effective excitation wavelength shifts to 345 nm. This property is used to measure local Ca^{2+} concentrations within cells. Cells can be loaded with fura-2 esters (e.g. fura-2AM) that diffuse across cell membranes and are hydrolyzed to active fura-2 by cytosolic esterases.

In the presence of 1mM Ca^{2+}, Trp8 expressing cells typically contained more than 300 nM cytosolic Ca^{2+}, while non transfected controls contained less than 100 nM Ca^{2+} ions (Fig. 3F).

When Trp8b transfected cells were incubated without extracellular Ca^{2+}, the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) decreased to levels comparable to non transfected cells. Readdition of 1mM Ca^{2+} to the bath resulted in significant increase of the cytosolic $[Ca^{2+}]$ in Trp8b transfected cells, but not in controls (Fig.: 3F). After readdition of Ca^{2+} ions to the bath solution, the cytosolic Ca^{2+} concentration remains on a high steady state level in the Trp8b transfected cells.

Example 5: Trp8 expressing cells show calcium selective inward currents

To characterize in detail the electrophysiological properties of TRP8, TRP8 and GFP were coexpressed in HEK293 cells using the dicistronic expression vector pdiTRP8 and measured currents using the patch clamp technique in the whole cell mode (Hamill, O.P., Marty, A., Neher, E., Sakmann, B. and Sigworth, F.J. (1981) Pflugers Arch., 391, 85-100).

The eucaryotic expression plasmid pdiTRP8 contains the cDNA of Trp8b under the control of the chicken ß-actin promotor followed by an internal ribosome entry side (IRES) and the cDNA of the green fluorescent protein (GFP). To obtain pdiTRP8 carrying the entire protein coding regions of TRP8b and the GFP (Prasher, D.C. et al. (1992), Gene 111, 229-233), the 5`and 3´-untranslated sequences of the TRP8b cDNA were removed, the consensus sequence for initiation of translation in vertebrates (Kozak, M. (1987) Nucleic Acids Research15, 8125-8148) was introduced immediately 5`of the translation initiation codon and the resulting cDNA was subcloned into the pCAGGS vector (Niwa, H., Yamamura, K. and Miyazaki, J (1991), Gene 8, 193-199) downstream of the chicken β-actin promotor. The IRES derived from encephalmyocarditis virus (Kim, D.G., Kang, H.M., Jang, S.K. and Shin H.S. (1992) Mol.Cell.Biol. 12, 3636-3643) followed by the GFP cDNA containing a Ser65Thr mutation (Heim, R., Cubitt, A.B., Tsien, R.Y. (1995) Nature 373, 663-664) was then cloned 3` to the TRP8b cDNA. The IRES sequence allows the simultaneous translation of TRP8b and GFP from one transcript. Thus, transfected cells can be detected unequivocally by the development of green fluorescence.

In the presence of 2 mM external calcium, Trp8b transfected HEK cells show inwardly rectifying currents, the size of which depends on the level of intracellular calcium and the electrochemical driving force. The resting membrane potential was held either at –40 mV, or, to lower the driving force for calcium influx in between pulses, at + 70 mV. Current traces

were recorded in response to voltage ramps from –100 to +100 mV, that were applied every second. To monitor inward and outward currents over time, we analyzed the current size at – 80 and + 80 mV of the ramps. Figure 3A shows a representative trace of the current at – 80 mV over time. Both at a holding potential of –40 mV or at +70 mV, the currents are significantly larger than in cells transfected with only the GFP containing vector (Fig.: 3E). Interestingly, after changing to a positive holding potential, current size in Trp8 transfected cells slowly increases and reaches steady state after approximately 70 seconds (Fig.: 3A). To determine the selectivity of the induced currents, we then perfused the cells with solutions that either contain no sodium, no added Ca^{2+} (Fig. 3A, C) or a sodium containing, but divalent ion free bath solution. To control for the effect of the solution change alone, we also perfused with normal bath (see puff in Fig. 3A). While removal of external Ca^{2+} completely abolishes the trp 8 induced currents - the remaining current being identical in size and shape to the control (Fig.: 3A, C, E), removal of external sodium has no effect (Fig.: 3E). An important hallmark of calcium selective channels (e.g. Vennekens, R., Hoenderop, G.J., Prenen, J., Stuiover, M., Willems, PHGM, Droogmans, G., Nilius, B.and Bindels, R.J.M (1999) J. Biol. Chem. 275, 3963-3969), is their ability to conduct sodium only if all external divalent ions, namely Ca^{2+} and magnesium are removed. To test whether the trp 8 channel conforms with this phenomenon normal bath solution was switched to a solution containing only sodium and 1 mM EGTA. As can be seen in Figure 3B and D, Trp8 transfected cells can now conduct very large sodium currents. Interestingly, immediately after the solution change, the currents first become smaller before increasing rapidly, indicating that the pore may initially still be blocked by calcium a phenomenon usually called anomalous mole fraction behaviour (Warnat, J., Philipp, S., Zimmer, S.,.Flockerzi, V., and Cavalié A.(1999) J.Physiol. (Lond) 518, 631-638). The measured outward currents of Trp8 transfected cells in normal bath solution are not significantly different from non-transfected control cells or cells which only express the reporter gene GFP. As the removal of external Ca^{2+} abolishes the Trp8 specific current, the remaining current was subtracted from the current before the solution change to obtain the uncontaminated Trp8 conductance (see inset in Fig.: 3C). As expected from the given ionic conditions (high EGTA inside, 2 mM Ca^{2+} outside), the current-voltage relationship now shows prominent inward rectification with little to no outward current.

Both the time course of the development of Trp8 currents and the size of the currents depend on the frequency of stimulation (data not shown), the internal and external Ca^{2+} concentration

and the resting membrane potential, suggesting that Trp8 calcium conductance is intrically regulated by a Ca^{2+} mediated feedback mechanisms.

Example 6: Ca^{2+} / calmodulin binds to the C-terminus of the Trp8 protein

To test whether calmodulin, a prime mediator of calcium regulated feedback, is involved, first it was investigated biochemically whether Trp8 protein can bind calmodulin. Trp8 cDNA was in vitro translated in the presence of ^{35}S-methionine and the product incubated with calmodulin-agarose beads. After several washes either in the presence or abscence of Ca^{2+}, the beads were incubated in Laemmli buffer and subjected to SDS-polyacrylamide gel electrophoresis. In the presence of Ca^{2+} (1mM), but not in the absence of Ca^{2+}, Trp8 protein binds to calmodulin (Fig.: 4B).

To narrow down the binding site, two approaches were undertaken: Firstly, GST-TRP8 fusion proteins of various intracellular domains of Trp8 were constructed, expressed in E. coli and bound to gluthathione sepharose beads. These beads were then incubated with in vitro translated ^{35}S- labeled calmodulin, washed and subjected to gel electrophoresis. Secondly, truncated versions of in vitro translated Trp8 protein were used in the above described binding to calmodulin-agarose. As shown in Figure 4A, and C, fusion proteins of the N-terminal region (N1, N2) of Trp8 did not bind calmodulin, while C-terminal fragments (C1, C2, C3, C4) showed calmodulin binding in the presence of calcium (for localization of fragments within the entire Trp8 protein see Fig. 4C). Accordingly, a truncated version of in vitro translated Trp8, which lacks the C-terminal 32 amino acid residues did not bind to calmodulin-agarose (4B). We have restricted the calmodulin binding site to amino acid residues 691 to 711 of the Trp8 protein. This calmodulin binding site does not resemble the typical conserved IQ - motif of conventional myosins, but has limited sequence homology to the calcium dependent calmodulin binding site 1 of the transient receptor potential like (trpl) protein of Drosophila melanogaster (Warr and Kelly, 1996) with several charged amino acid residues conserved. The sequence of the calmodulin binding site of the Trp8 protein resembles a putative amphipathic α-helical wheel structure with a charged and a hydrophobic site according to a model proposed by Erickson-Vitanen and De Grado (1987, Methods Enzymol. 139, 455-478.).

Example 7: Expression of Trp8 transcripts in human placenta and pancreas

Several slides from a human placenta of a ten week old abort were used for in situ hybridization experiments. The in situ hybridization experiments revealed expression of Trp8 transcripts in human placenta (Fig.: 5B). Expression was detectable in trophoblasts and syncytiotrophoblasts of the placenta, but not in Langhans cells.

Trp8 transcripts are detectable in human pancreas (Fig.: 5A). Therefore Trp8 probes were hybridized to tissue sections of human pancreas. The pancreatic tissues were removed from patients with pancreas cancer. Trp8 expression is detectable in pancreatic acinar cells, but not in Langerhans islets (Fig.: 5C). No Trp8 expression was found in regions of pancreatic carcinomas (data not shown).

Furthermore, the Trp8 cDNA is not detectable in human colon nor in human kidney by in situ hybridization as well as by Northern analysis (Fig.: 5A, D). The Northern results taken together with the in situ expression data indicate that the Trp8 protein is not the human ortholog of the CaT1 and ECaC channels cloned from rat intestine (Peng, J.B., Chen, X.Z., Berger, U.V., Vassilev, P.M., Tsukaguchi, H., Brown, E.M. and Hediger M.A.(1999) J Biol Chem. 6;274, 22739-22746) and from rabbit kidney (Hoenderop, J.G., van der Kemp, A.W., Hartog, A., van de Graaf, S.F., van Os, C.H., Willems, P.H. and Bindels, R.J. (1999) J Biol Chem. 26;274, 8375-8378), respectively. Trp8 is unlikely to represent the human version of CaT1 as its expression is undetectable in the small intestine and colon tissues where CaT1 is abundantly expressed. If, however, Trp8 is the human version of rat CaT1, a second gene product appears to be required for Ca^{2+} uptake in human small intestine and colon attributed to CaT1 in rat small intestine and colon.

Example 8: Differential expression of Trp8 transcripts in benign and malign tissue of the prostate

The Trp8 transcripts are expressed in human prostate as shown by hybridization of a Trp8 probe to a commercial Northern blot (Clontech, Palo Alto, USA) (Fig.: 5A). Trp8 transcripts were not detectable by Northern blot analysis using pooled mRNA of patients with benign prostatic hyperplasia (BPH) (Fig.: 5A, prostate*). To examine Trp8 expression on the cellular

level, sections of prostate tissues were hybridized using Trp8 specific cDNA probes (Table 3). Expression of Trp8 transcripts is not detectable in normal prostate (n = 3), benign hyperplasia (BPH, n = 15) or prostatic intraepithelial neoplasia (PIN, n = 9) (Fig.: 6A, C, E). Trp8 transcripts were only detectable in prostate carcinoma (PCA), although with different expression levels. Low expression levels were found in primary carcinomas (2 - 10 % of the carcinoma cells, n = 8) (Fig.: 7B) . Much stronger expression was detectable in rezidive carcinoma (10 - 60 %) (Fig.: 7D, n = 6) and metastases of the prostate (60 - 90 %, n = 4) (Fig.: 7F). Thus it has to be concluded that the commercial Northern blot used in Fig.: 5A contains not only normal prostate mRNA as indicated by the distributor. According to the distributors instructions the prostate mRNA used for this Northern blot was collected from 15 human subjects in the range of 14 to 60 years of age. This prostate tissue was not examined by pathologic means. Since Trp8 expression is not detectable in normal or benign prostate, this finding implicates that the mRNA used for this Northern blot was extracted in part from prostatic carcinoma tissue. To summarize, Trp8 expression is only detectable in malign prostate and, thus, the Trp8 cDNA is a marker for prostate carcinoma. The results are summarized in Table 4.

Table 3

Trp8 probes used for in situ hybridization:

Probes (antisense)

1.) 5' TCCGCTGCCGGTTGAGATCTTGCC 3'

2.) 5' CTTGCTCCATAGGCAGAGAATTAG 3'

3.) 5' ATCCTCAGAGCCCCGGGTGTGGAA3'

Controls (sense)

1.) 5' GGCAAGATCTCAACCGGCAGCGGA 3'

2.) 5' CTAATTCTCTGCCTATGGAGCAAG 3'

3.) 5' TTCCACACCCGGGGCTCTGAGGAT 3'

Table 4

Prostate	total	negative	positive
normal	3	3	0
BPH	15	15	0
PIN	9	9	0

carcinoma	18	1	17

(B) Differential expression of Trp8 transcripts in benign and malign tissue of the uterus

Moreover it could be shown that Trp8 is expresed in endometrial cancer (also called cancer of the uterus, to be distinguished from uterine sarcoma or cancer of the cervix) whereas no expression was observed in normal uterus tissue. Thus, Trp8 also is a specific marker for the diagnosis of the above cancer (Fig. 12).

Example 9: Characterization of Trp9

The complete protein coding sequence of Trp9 was determined (Fig. 9). Trp 9 transcripts are predominantly expressed in the human prostate and in human colon. As it could be shown by Northern blot analysis, there is no difference of the expression of TRP9 in benigne prostata hyperplasia (BPH, Fig. 13, upper panel left) or prostate carcinoma (Fig. 13, upper panel right). However, Trp9 is useful as a reference marker for prostata carcinoma, i.e. can be used for quantifying the expression level of Trp8. The ratio of the expression of Trp8:Trp9 in patients and healthy individuals is useful for the development of a quantitative assay.

Example 10: Characterization of Trp10

The complete protein coding sequence of TRP10 (a and b) was determined by biocomputing (Fig. 10 and 11). Using a 235 bp fragment of the Trp10 cDNA as probe in Northern blot analysis TRP10 transcripts could only be detected in mRNA isolated from individuals with prostate cancer (Fig. 13, bottom panel) but not in mRNA isolated from benign tissue of the prostate (prostate BPH) nor in mRNA isolated from heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas. The 235 bp cDNA fragment of the Trp10 cDNA was amplified using the primer pair UW248 5'-ACA GCT GCT GGT CTA TTC C-3' and UW249 5'-TAT

GTG CCT TGG TTT GTA CC-3' and prostate cDNA as template. In summary, Trp10a and Trp10b , like TRP8 are also expressed in malignant prostate tissue. So far, its expression could not be observed in any other tissue examined (see above). Thus, Trp 10a and Trp10b are also useful markers which are specific for malignant prostate tissue.

Furthermore, database searches in public databases of the national center for biological information (NCBI) revealed the existence of several expressed sequence tags (EST clones) being in part identical to the Trp10 sequence. These EST clones were originally isolated from cancer tissues of lung, placenta, prostate and from melanoma. These clones include the clones with the following accession numbers: BE274448, BE408880, BE207083, BE791173, AI671853, BE390627. The results demonstrate that cancer cells of these tissues express Trp10 related transcripts whereas no expression of Trp10 transcripts in the corresponding healthy tissues are detectable (Figure 13). Furthermore, it could be shown that in cancer cells of melanoma and prostate cancer Trp10 transcripts are expressed as shown by in situ hybridizations using 4 antisense probes (Figure 14A – E and 13K-O and Table 2, above). Furthermore, it could clearly be shown that cancer cells of these tissues expressing Trp10 transcripts also express Trp10-antisense transcripts as shown in Figure 14F-J, Figure 14P-R and Figure14T by in situ hybridizations using 4 sense probes (Table 2, above). The in situ hybridization experiments demonstrate that detection of a subset of cancer cells derived from carcinoma of lung, placenta, prostate and melanoma is feasible using antisense as well as sense probes complementary to Trp10 transcripts or complementary to Trp10-antisense transcripts, respectively.

The foregoing is meant to illustrate but not to limit the scope of the invention. The person skilled in the art can readily envision and produce further embodiment, based on the above teachings, without undue experimentation.

What Is claimed Is:

1. An isolated nucleic acid molecule encoding the human prostate carcinoma associated protein Trp8a, Trp8b, Trp9, Trp10a or Trp10b or a protein exhibiting biological properties of Trp8a, Trp8b, Trp9, Trp10a or Trp10b and being selected from the group consisting of

(a) a nucleic acid molecule encoding a protein that comprises the amino acid sequence depicted in Figure 7, 8A, 9, 10 or 11;

(b) a nucleic acid molecule comprising the nucleotide sequence depicted in Figure 7, 8A, 9, 10 or 11;

(c) a nucleic acid molecule included in DSMZ Deposit No. DSM 13579, DSM 13580, DSM 13584 , DSM 13581 or DSM....;

(d) a nucleic acid molecule which hybridizes to a nucleic acid molecule specified in (a) to (c);

(e) a nucleic acid molecule the nucleic acid sequence of which deviates from the nucleic sequences specified in (a) to (d) due to the degeneration of the genetic code; and

(f) a nucleic acid molecule, which represents a fragment, derivative or allelic variation of a nucleic acid sequence specified in (a) to (e).

2. A recombinant vector containing the nucleic acid molecule of claim 1

3. The recombinant vector of claim 2 wherein the nucleic acid molecule is operatively linked to regulatory elements allowing transcription and synthesis of a translatable RNA in prokaryotic and/or eukaryotic host cells.

4. A recombinant host cell which contains the recombinant vector of claim 3.

5. The recombinant host cell of claim 4, which is a mammalian cell, a bacterial cell, an insect cell or a yeast cell.

6. An isolated protein exhibiting biological properties of the human prostate carcinoma associated protein Trp8a, Trp8b, Trp9, Trp10a or Trp10b which is encoded by a nucleic acid molecule of claim 1.

7. A recombinant host cell that expresses the isolated protein of claim 6.

8. A method of making an isolated protein exhibiting biological properties of the human prostate carcinoma associated protein Trp8a, Trp8b, Trp9, Trp10a or Trp10b comprising:
(a) culturing the recombinant host cell of claim 6 under conditions such that said protein is expressed; and
(b) recovering said protein.

9. The protein produced by the method of claim 8.

10. An antisense RNA sequence characterized in that it is complementary to an mRNA transcribed from a nucleic acid molecule of claim 1 or a part thereof and can selectively bind to said mRNA or part thereof, said sequence being capable of inhibiting the synthesis of the protein encoded by said nucleic acid molecule.

11. A ribozyme characterized in that it is complementary to an mRNA transcribed from a nucleic acid molecule of claim 1 or a part thereof and can selectively bind to and cleave said mRNA or part thereof, thus inhibiting the synthesis of the protein encoded by said nucleic acid molecule.

12. An inhibitor characterized in that it can suppress the activity of the protein of claim 6.

13. A method for diagnosing a prostate carcinoma which comprises contacting a target sample suspected to contain the protein Trp8a, Trp8b, Trp10a and/or Trp10b or the Trp8a, Trp8b, Trp10a and/or Trp10b encoding mRNA with a reagent which reacts with Trp8a, Trp8b, Trp10a and/or Trp10b or the Trp8a, Trp8b, Trp10a and/or Trp10b encoding mRNA and detecting Trp8a, Trp8b, Trp10a and/or Trp10b or the Trp8a, Trp8b, Trp10a and/or Trp10b encoding mRNA.

14. The method of claim 13, wherein the reagent is a nucleic acid.

15. The method of claim 13, wherein the reagent is an antibody.

16. The method of claim 13, wherein the reagent is detectably labeled.

17. The method of claim 16, wherein the label is selected from the group consisting of a radioisotope, a bioluminescent compound, a chemiluminescent compound, a fluorescent compound, a metal chelate, or an enzyme.

18. A method for diagnosing an endometrial cancer (carcinoma of the uterus) which comprises contacting a target sample suspected to contain the protein Trp8a and/or Trp8b or the Trp8a and/or Trp8b encoding mRNA with a reagent which reacts with Trp8a and/or Trp8b or the Trp8a and/or Trp8a and/or trp8b encoding mRNA and detecting Trp8a and/or Trp8b or the Trp8a and/or Trp8b encoding mRNA.

19. The method of claim 18, wherein the reagent is a nucleic acid.

20. The method of claim 18, wherein the reagent is an antibody.

21. The method of claim 18, wherein the reagent is detectably labeled.

22. The method of claim 21, wherein the label is selected from the group consisting of a radioisotope, a bioluminescent compound, a chemiluminescent compound, a fluorescent compound, a metal chelate, or an enzyme.

23. A method for diagnosing a melanoma, chorion carcinoma, cancer of the lung and of the prostate in a tissue of a subject, comprising contacting a sample with a reagent which detects Trp10a and/or Trp10b antisense RNA or Trp10a and/or Trp10b related antisense RNA.

24. A method for preventing, treating, or ameliorating a prostate tumor, endometrial cancer (carcinoma of the uterus) tumor, a chorion carcinoma, cancer of the lung or melanoma, which comprises administering to a mammalian subject a therapeutically effective amount of a reagent which decreases or inhibits expression of Trp8a, Trp8b, Trp10a and/or Trp10b and/or the activity of Trp8a, Trp8b, Trp10a and/or Trp10b.

25. The method of claim 24, wherein the reagent is a nucleotide sequence comprising an antisense RNA.

26. The method of claim 24, wherein the reagent is a nucleotide sequence comprising a ribozyme.

27. The method of claim 24, wherein the reagent is an inhibitor of Trp8a, Trp8b, Trp10a and/or Trp10b.

28. The method of claim 27, wherein the reagent is an anti-Trp8a-, anti Trp8b-, anti-Trp10a- and/or anti-Trp10b antibody or a fragment thereof.

29. A diagnostic kit useful for the detection of Trp8a, Trp8b, Trp10a and/or Trp10b or Trp8a, Trp8b, Trp10a and/or Trp10b encoding mRNA or Trp10a and/or Trp10b antisense transcripts in a sample, wherein the presence of an increased concentration of Trp8a, Trp8b, Trp9, Trp10a and/or Trp10b or Trp8a, Trp8b, Trp9, Trp10a and/or Trp10b encoding mRNA or Trp10a and/or Trp10b antisense transcripts is indicative for a prostate tumor, endometrial cancer (cancer of the uterus) tumor, a chorion carcinoma, cancer of the lung or melanoma, said kit comprising a probe for detection of Trp8a, Trp8b, Trp9, Trp10a or Trp10b or Trp8a, Trp8b, Trp9, Trp10a and/or Trp10b encoding mRNA or Trp10a and/or Trp10b antisense transcripts.

30. The kit of claim 29, wherein the target component to be detected is Trp8a, Trp8b, Trp9, Trp10a and/or Trp10b and the probe is an antibody.

31. A method for identifying a compound which acts as an agonist or antagonist on the ion channels Trp8, Trp9 and/or Trp10, said method comprising contacting a test compound with the ion channel Trp8, Trp9 and/or Trp10,and determining whether said test compound affects the calcium uptake.

Figs. 1A and 1B

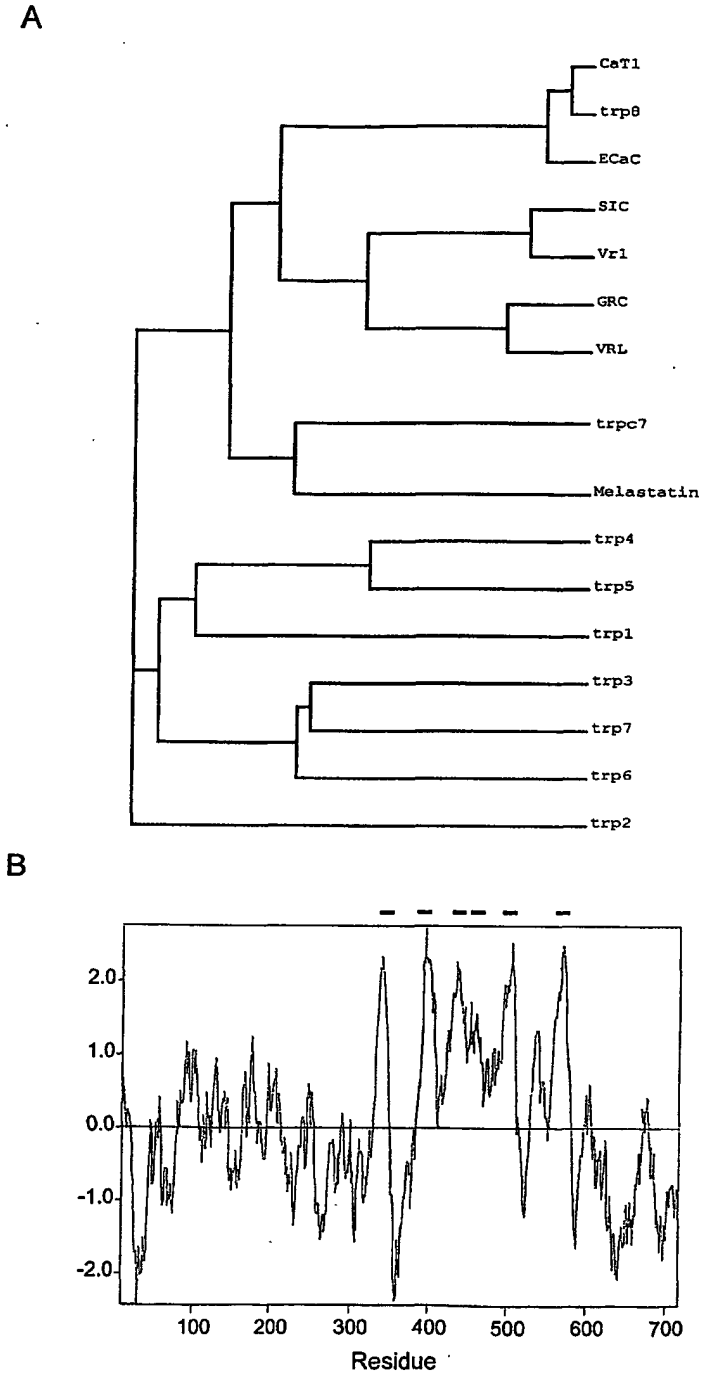
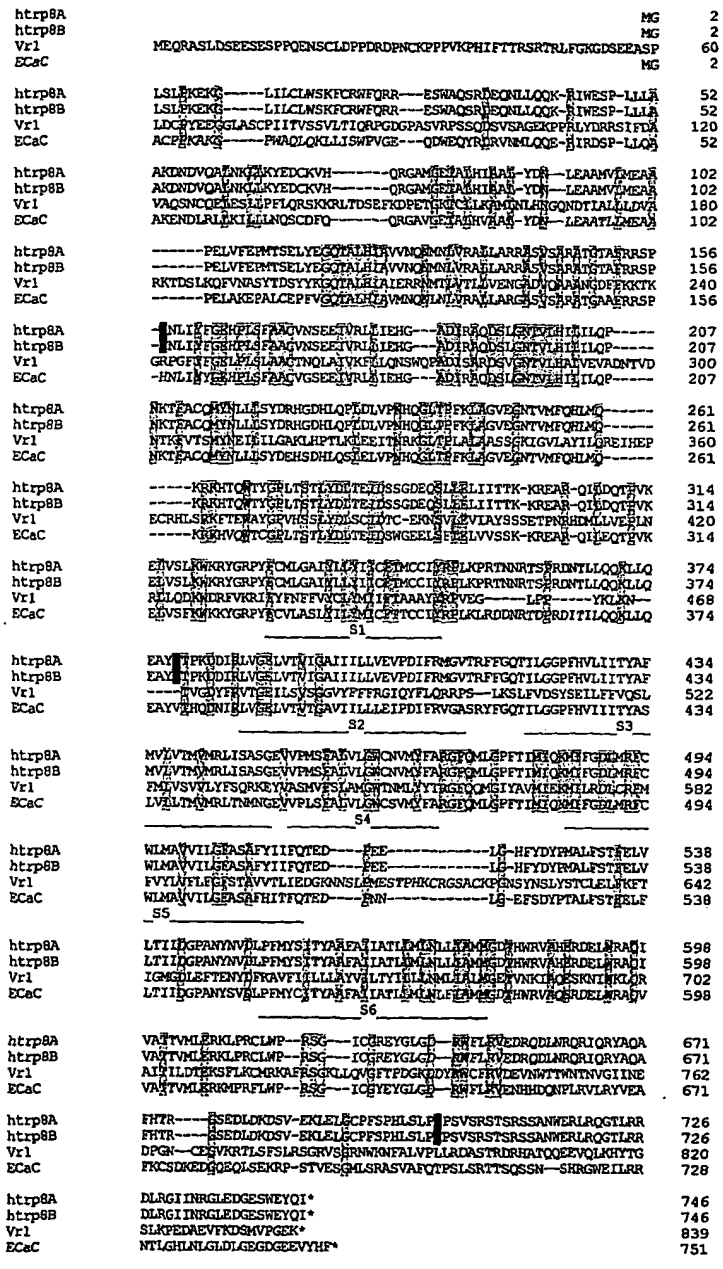
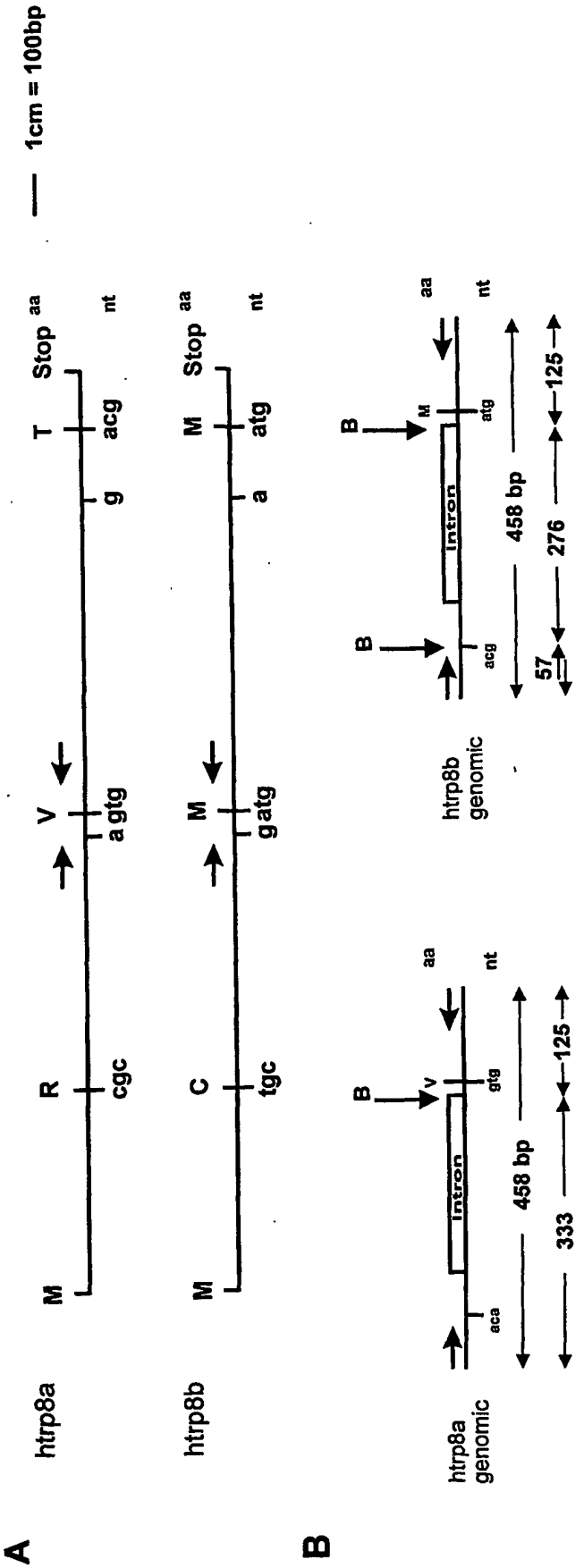


Fig. 1C

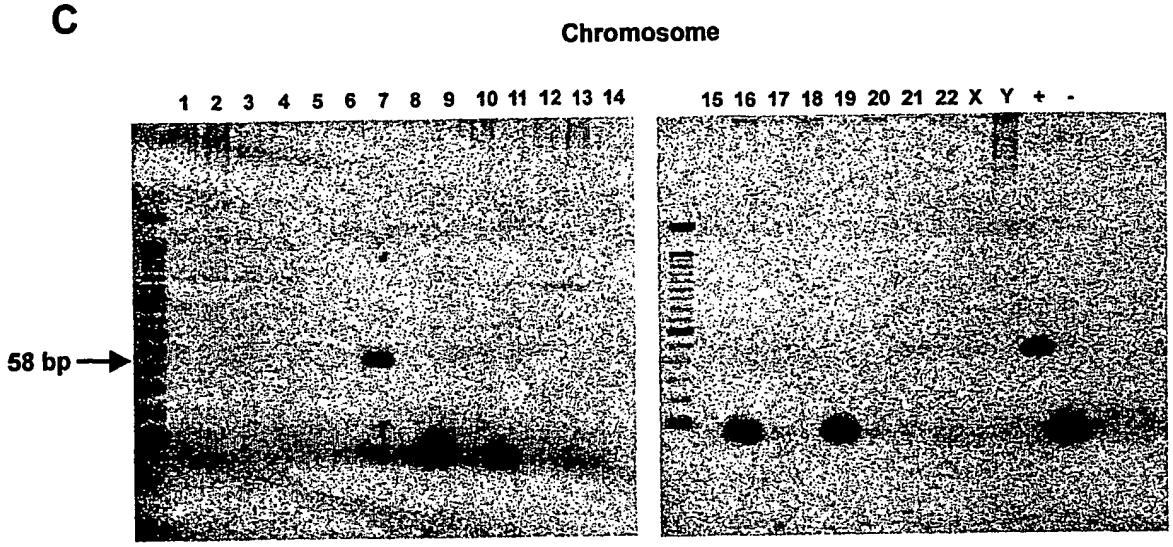
C

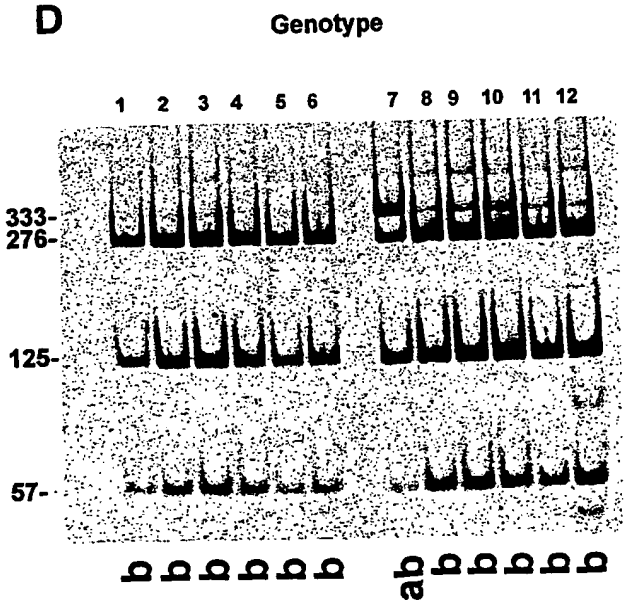




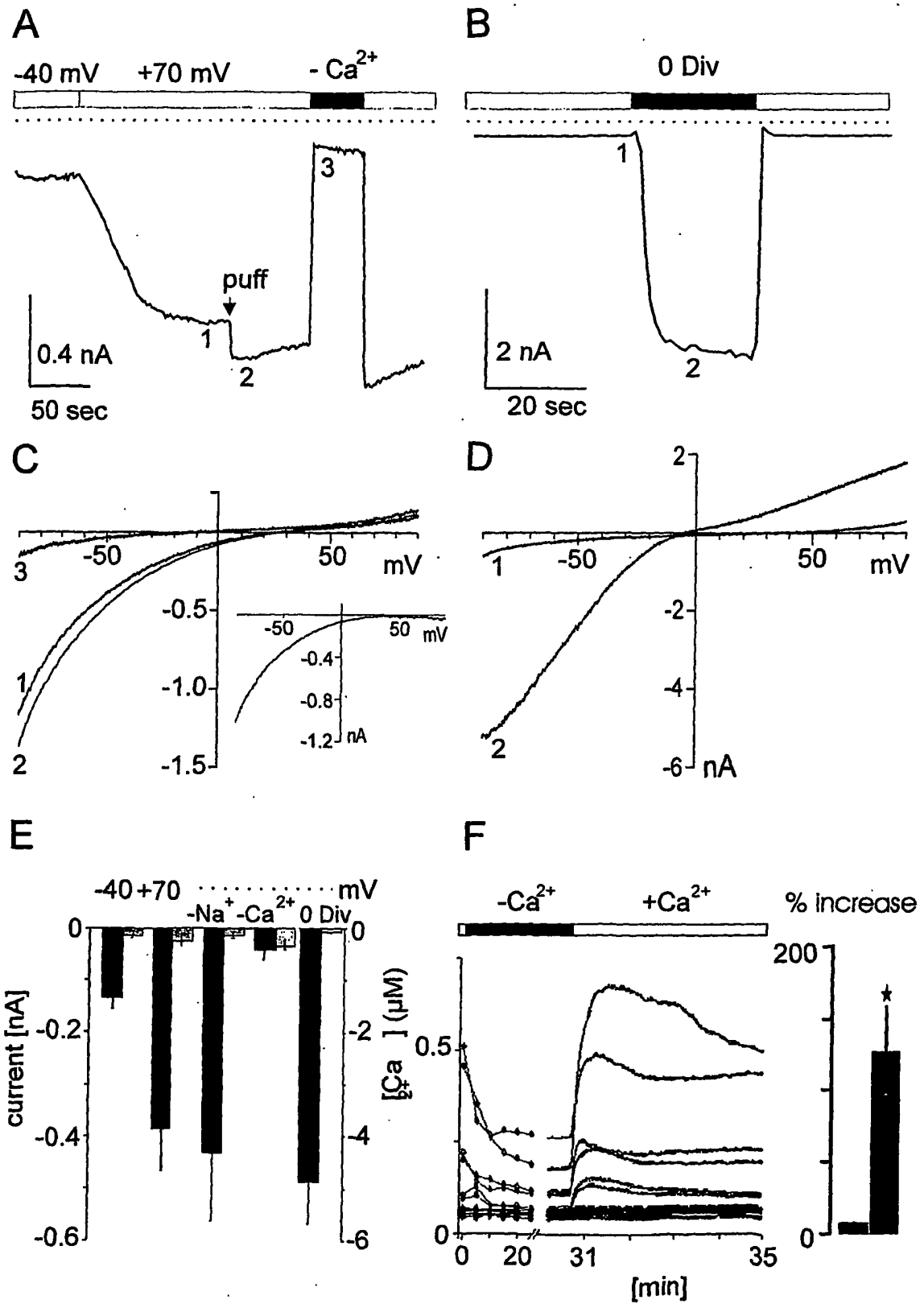
Figs. 2A and 2B

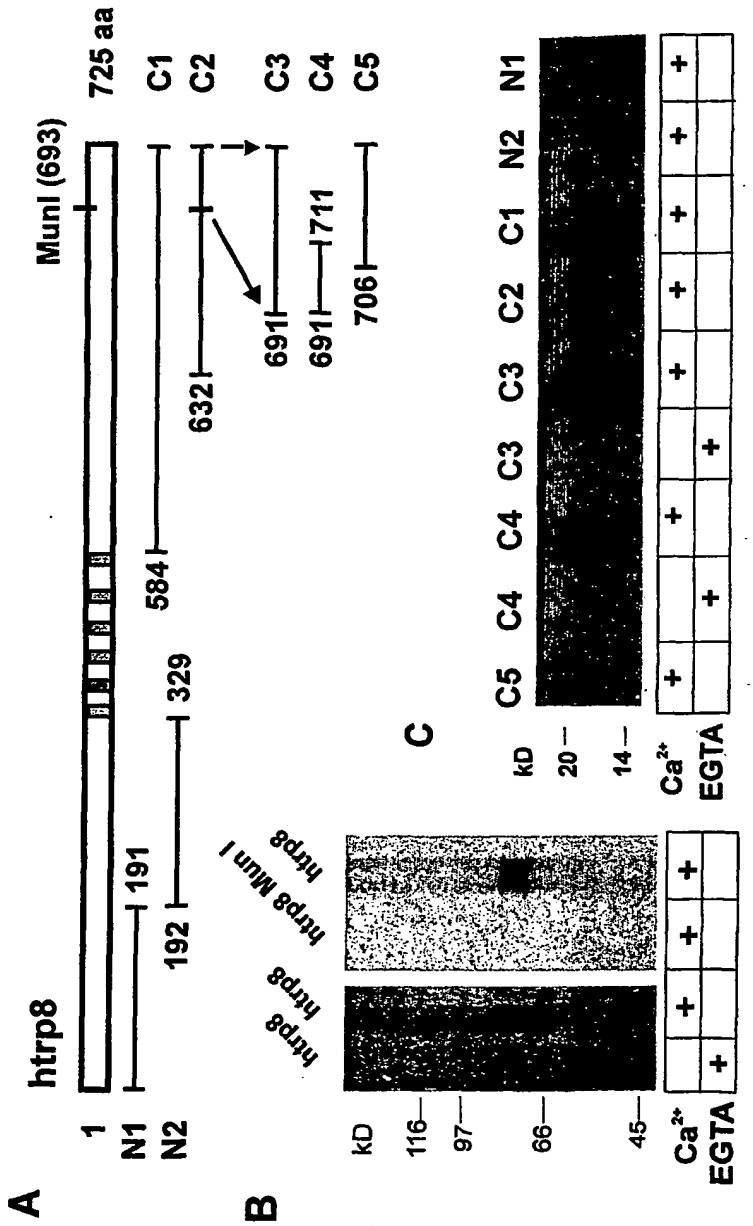
Figs. 2C and 2D





Figs. 3A – 3F


A
-40 mV
+70 mV
- Ca2+
puff
0.4 nA
50 sec
1
2
3
B
0 Div
2 nA
20 sec
C
-50
50
mV
-0.5
-1.0
-1.5
-0.4
-0.8
-1.2
nA
D
-2
-4
-6
E
-40 +70 mV
-Na+ -Ca2+ 0 Div
current [nA]
-0.2
-0.4
-0.6
F
-Ca2+
+Ca2+
% increase
200
[Ca2+] (µM)
0.5
0
20
31
35
[min]



Figs. 4A – 4C

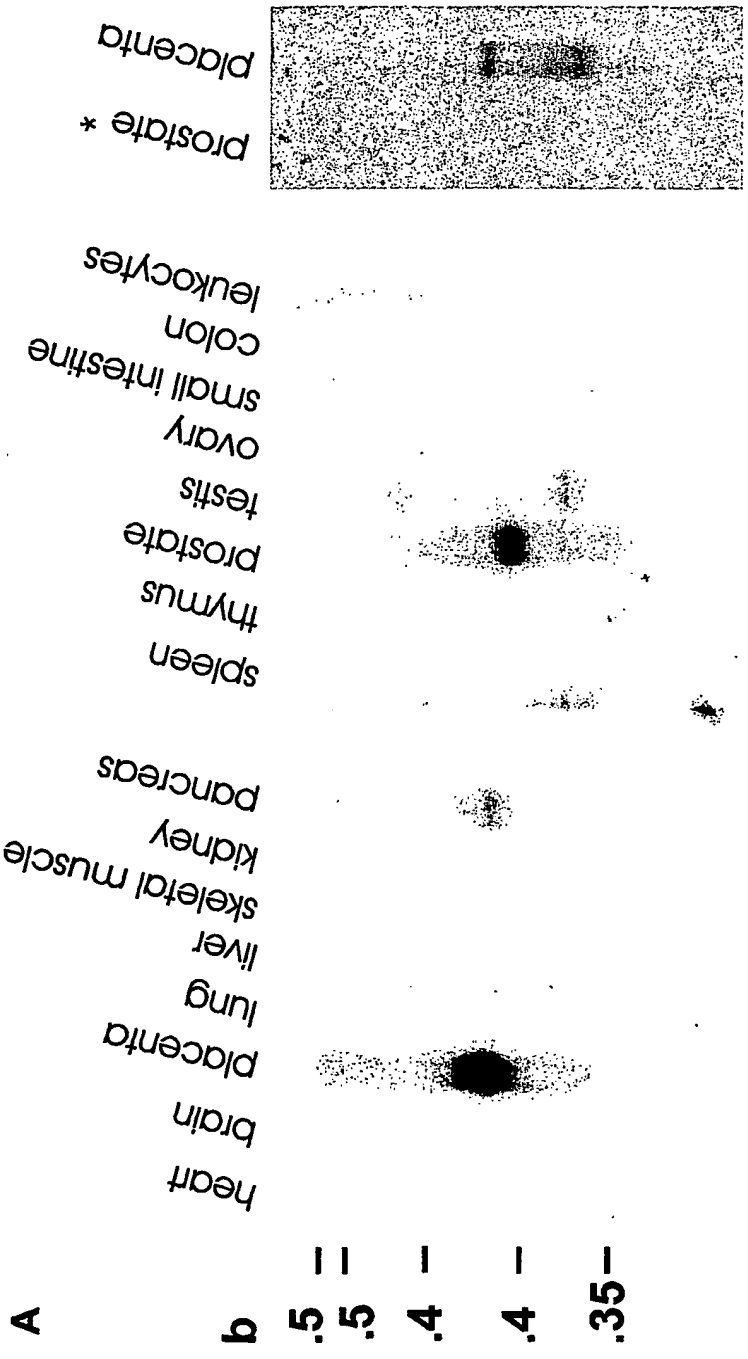
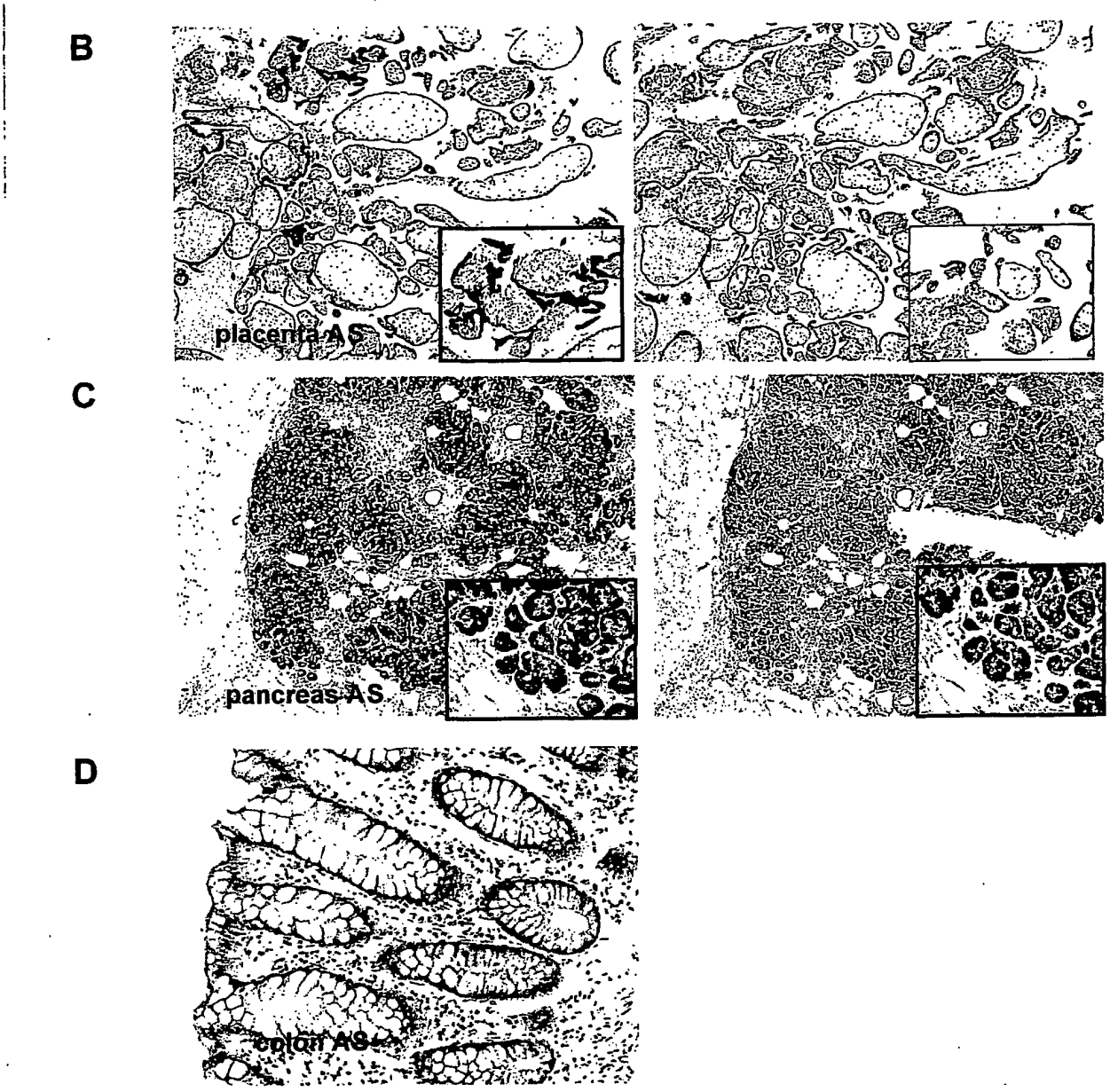
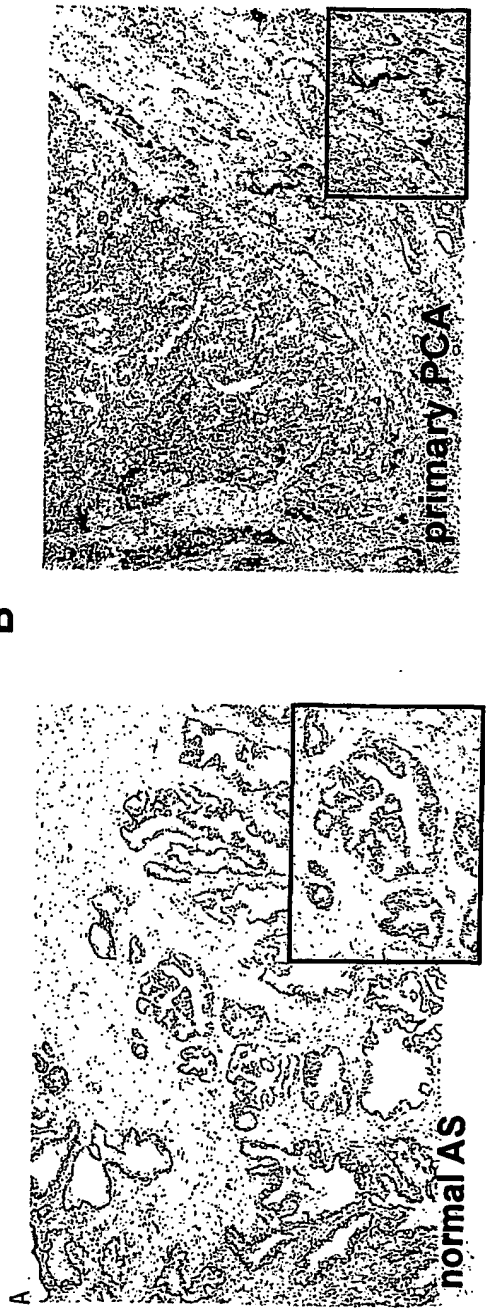


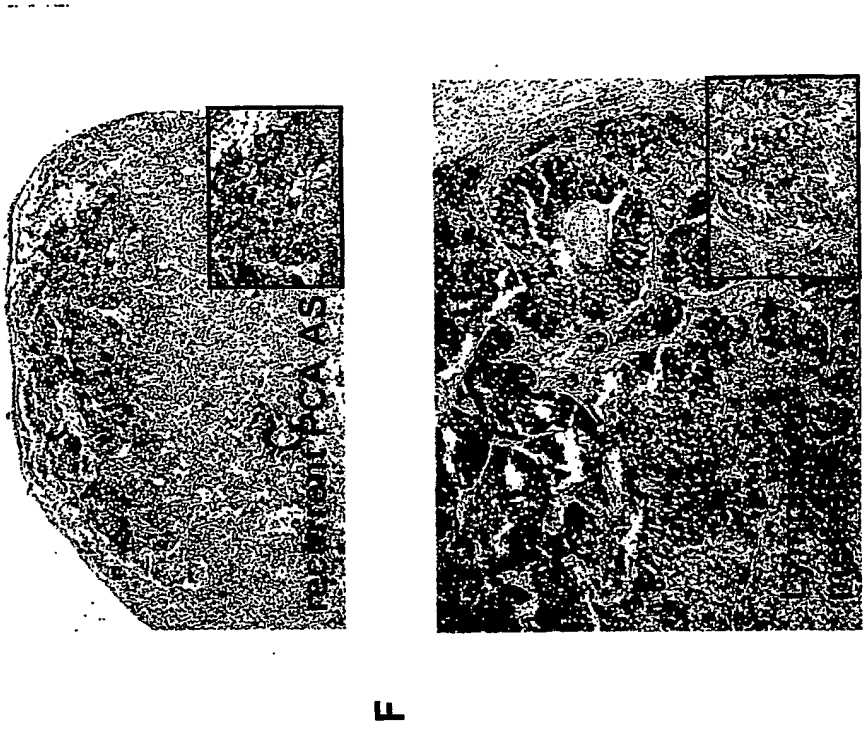
Fig. 5A

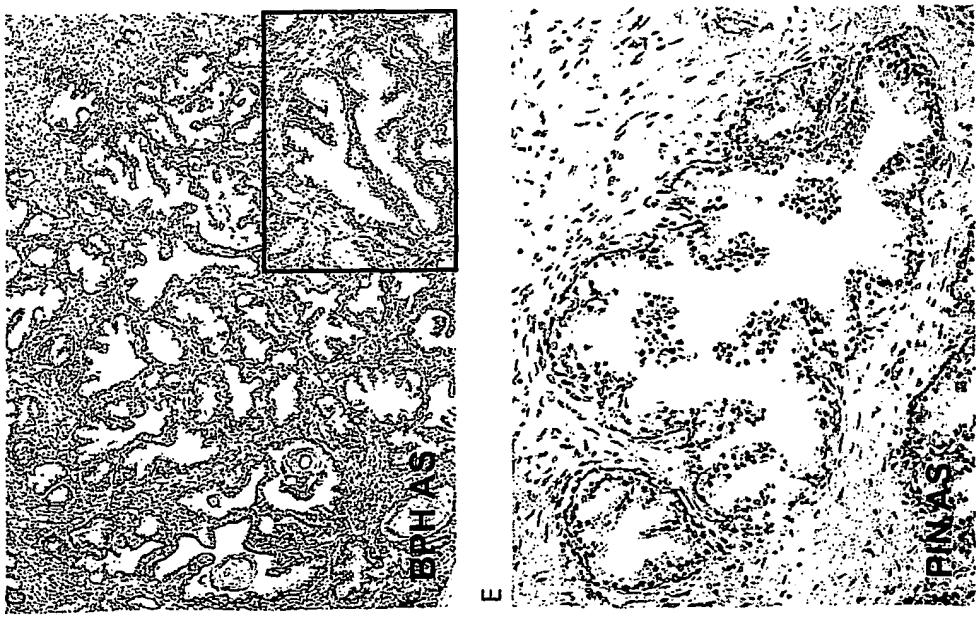
Figs. 5B – 5D





Figs. 6A and 6B





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Figs. 6C – 6F

Fig. 7

```
        10                  30                  50
GCCAAGTGTAACAAACTCACAGCCCTCTCCAAACTGGCTGGGGCTGCTGGGAGACTCCCA
        70                  90                  110
AGGAACTCGTCAGGAAGGCAGGAGACAGGAGACGGGACCTCTACAGGGAGACGGTGGGCC
       130                 150                 170
GGCCCTTGGGGGGGCTGATGTGGCCCCAAGGCTGAGTCCCGTCAGGGTCTGGCCTCGGCC
       190                 210                 230
TCAGGCCCCCAAGGAGCCGGCCCTACACCCCATGGGTTTGTCACTGCCCAAGGAGAAAGG
                                M  G  L  S  L  P  K  E  K  G
       250                 270                 290
GCTAATTCTCTGCCTATGGAGCAAGTTCTGCAGATGGTTCCAGAGACGGGAGTCCTGGGC
 L  I  L  C  L  W  S  K  F  C  R  W  F  Q  R  R  E  S  W  A
       310                 330                 350
CCAGAGCCGAGATGAGCAGAACCTGCTGCAGCAGAAGAGGATCTGGGAGTCTCCTCTCCT
 Q  S  R  D  E  Q  N  L  L  Q  Q  K  R  I  W  E  S  P  L  L
       370                 390                 410
TCTAGCTGCCAAAGATAATGATGTCCAGGCCCTGAACAAGTTGCTCAAGTATGAGGATTG
 L  A  A  K  D  N  D  V  Q  A  L  N  K  L  L  K  Y  E  D  C
       430                 450                 470
CAAGGTGCACCAGAGAGGAGCCATGGGGGAAACAGCGCTACACATAGCAGCCCTCTATGA
 K  V  H  Q  R  G  A  M  G  E  T  A  L  H  I  A  A  L  Y  D
       490                 510                 530
CAACCTGGAGGCCGCCATGGTGCTGATGGAGGCTGCCCCGGAGCTGGTCTTTGAGCCCAT
 N  L  E  A  A  M  V  L  M  E  A  A  P  E  L  V  F  E  P  M
       550                 570                 590
GACATCTGAGCTCTATGAGGGTCAGACTGCACTGCACATCGCTGTTGTGAACCAGAACAT
 T  S  E  L  Y  E  G  Q  T  A  L  H  I  A  V  V  N  Q  N  M
       610                 630                 650
GAACCTGGTGCGAGCCCTGCTTGCCCGCAGGGCCAGTGTCTCTGCCAGAGCCACAGGCAC
 N  L  V  R  A  L  L  A  R  R  A  S  V  S  A  R  A  T  G  T
       670                 690                 710
TGCCTTCCGCCGTAGTCCCCGCAACCTCATCTACTTTGGGGAGCACCCTTTGTCCTTTGC
 A  F  R  R  S  P  R  N  L  I  Y  F  G  E  H  P  L  S  F  A
       730                 750                 770
TGCCTGTGTGAACAGTGAGGAGATCGTGCGGCTGCTCATTGAGCATGGAGCTGACATCCG
 A  C  V  N  S  E  E  I  V  R  L  L  I  E  H  G  A  D  I  R
       790                 810                 830
GGCCCAGGACTCCCTGGGAAACACAGTGTTACACATCCTCATCCTCCAGCCCAACAAAAC
 A  Q  D  S  L  G  N  T  V  L  H  I  L  I  L  Q  P  N  K  T
       850                 870                 890
CTTTGCCTGCCAGATGTACAACCTGTTGCTGTCCTACGACAGACATGGGGACCACCTGCA
 F  A  C  Q  M  Y  N  L  L  L  S  Y  D  R  H  G  D  H  L  Q
       910                 930                 950
GCCCCTGGACCTCGTGCCCAATCACCAGGGTCTCACCCCTTTCAAGCTGGCTGGAGTGGA
 P  L  D  L  V  P  N  H  Q  G  L  T  P  F  K  L  A  G  V  E
       970                 990                1010
GGGTAACACTGTGATGTTTCAGCACCTGATGCAGAAGCGGAAGCACACCCAGTGGACGTA
 G  N  T  V  M  F  Q  H  L  M  Q  K  R  K  H  T  Q  W  T  Y
      1030                1050                1070
TGGACCACTGACCTCGACTCTCTATGACCTCACAGAGATCGACTCCTCAGGGGATGAGCA
 G  P  L  T  S  T  L  Y  D  L  T  E  I  D  S  S  G  D  E  Q
      1090                1110                1130
GTCCCTGCTGGAACTTATCATCACCACCAAGAAGCGGGAGGCTCGCCAGATCCTGGACCA
 S  L  L  E  L  I  I  T  T  K  K  R  E  A  R  Q  I  L  D  Q
      1150                1170                1190
GACGCCGGTGAAGGAGCTGGTGAGCCTCAAGTGGAAGCGGTACGGGCGGCCGTACTTCTG
 T  P  V  K  E  L  V  S  L  K  W  K  R  Y  G  R  P  Y  F  C
      1210                1230                1250
CATGCTGGGTGCCATATATCTGCTGTACATCATCTGCTTCACCATGTGCTGCATCTACCG
 M  L  G  A  I  Y  L  L  Y  I  I  C  F  T  M  C  C  I  Y  R
      1270                1290                1310
```

```
CCCCCTCAAGCCCAGGACCAATAACCGCACAAGCCCCCGGGACAACACCCTCTTACAGCA
 P  L  K  P  R  T  N  N  R  T  S  P  R  D  N  T  L  L  Q  Q
      1330              1350              1370
GAAGCTACTTCAGGAAGCCTACGTGACCCCTAAGGACGATATCCGGCTGGTCGGGGAGCT
 K  L  L  Q  E  A  Y  V  T  P  K  D  D  I  R  L  V  G  E  L
      1390              1410              1430
GGTGACTGTCATTGGGGCTATCATCATCCTGCTGGTAGAGGTTCCAGACATCTTCAGAAT
 V  T  V  I  G  A  I  I  I  L  L  V  E  V  P  D  I  F  R  M
      1450              1470              1490
GGGGGTCACTCGCTTCTTTGGACAGACCATCCTTGGGGGCCCATTCCATGTCCTCATCAT
 G  V  T  R  F  F  G  Q  T  I  L  G  G  P  F  H  V  L  I  I
      1510              1530              1550
CACCTATGCCTTCATGGTGCTGGTGACCATGGTGATGCGGCTCATCAGTGCCAGCGGGGA
 T  Y  A  F  M  V  L  V  T  M  V  M  R  L  I  S  A  S  G  E
      1570              1590              1610
GGTGGTACCCATGTCCTTTGCACTCGTGCTGGGCTGGTGCAACGTCATGTACTTCGCCCG
 V  V  P  M  S  F  A  L  V  L  G  W  C  N  V  M  Y  F  A  R
      1630              1650              1670
AGGATTCCAGATGCTAGGCCCCTTCACCATCATGATTCAGAAGATGATTTTTGGCGACCT
 G  F  Q  M  L  G  P  F  T  I  M  I  Q  K  M  I  F  G  D  L
      1690              1710              1730
GATGCGATTCTGCTGGCTGATGGCTGTGGTCATCCTGGGCTTTGCTTCAGCCTTCTATAT
 M  R  F  C  W  L  M  A  V  V  I  L  G  F  A  S  A  F  Y  I
      1750              1770              1790
CATCTTCCAGACAGAGGACCCCGAGGAGCTAGGCCACTTCTACGACTACCCCATGGCCCT
 I  F  Q  T  E  D  P  E  E  L  G  H  F  Y  D  Y  P  M  A  L
      1810              1830              1850
GTTCAGCACCTTCGAGCTGTTCCTTACCATCATCGATGGCCCAGCCAACTACAACGTGGA
 F  S  T  F  E  L  F  L  T  I  I  D  G  P  A  N  Y  N  V  D
      1870              1890              1910
CCTGCCCTTCATGTACAGCATCACCTATGCTGCCTTTGCCATCATCGCCACACTGCTCAT
 L  P  F  M  Y  S  I  T  Y  A  A  F  A  I  I  A  T  L  L  M
      1930              1950              1970
GCTCAACCTCCTCATTGCCATGATGGGCGACACTCACTGGCGAGTGGCCCATGAGCGGGA
 L  N  L  L  I  A  M  M  G  D  T  H  W  R  V  A  H  E  R  D
      1990              2010              2030
TGAGCTGTGGAGGGCCCAGATTGTGGCCACCACGGTGATGCTGGAGCGGAAGCTGCCTCG
 E  L  W  R  A  Q  I  V  A  T  T  V  M  L  E  R  K  L  P  R
      2050              2070              2090
CTGCCTGTGGCCTCGCTCCGGGATCTGCGGACGGGAGTATGGCCTGGGGGACCGCTGGTT
 C  L  W  P  R  S  G  I  C  G  R  E  Y  G  L  G  D  R  W  F
      2110              2130              2150
CCTGCGGGTGGAAGACAGGCAAGATCTCAACCGGCAGCGGATCCAACGCTACGCACAGGC
 L  R  V  E  D  R  Q  D  L  N  R  Q  R  I  Q  R  Y  A  Q  A
      2170              2190              2210
CTTCCACACCCGGGGCTCTGAGGATTTGGACAAAGACTCAGTGGAAAAACTAGAGCTGGG
 F  H  T  R  G  S  E  D  L  D  K  D  S  V  E  K  L  E  L  G
      2230              2250              2270
CTGTCCCTTCAGCCCCCACCTGTCCCTTCCTACGCCCTCAGTGTCTCGAAGTACCTCCCG
 C  P  F  S  P  H  L  S  L  P  T  P  S  V  S  R  S  T  S  R
      2290              2310              2330
CAGCAGTGCCAATTGGGAAAGGCTTCGGCAAGGGACCCTGAGGAGAGACCTGCGTGGGAT
 S  S  A  N  W  E  R  L  R  Q  G  T  L  R  R  D  L  R  G  I
      2350              2370              2390
AATCAACAGGGGTCTGGAGGACGGGGAGAGCTGGGAATATCAGATCTGACTGCGTGTTCT
 I  N  R  G  L  E  D  G  E  S  W  E  Y  Q  I
      2410              2430              2450
CACTTCGCTTCCTGGAACTTGCTCTCATTTTCCTGGGTGCATCAAACAAAACAAAAACCA
      2470              2490              2510
AACACCCAGAGGTCTCATCTCCCAGGCCCCAGGGAGAAAGAGGAGTAGCATGAACGCCAA
      2530              2550              2570
GGAATGTACGTTGAGAATCACTGCTCCAGGCCTGCATTACTCCTTCAGCTCTGGGGCAGA
```

Fig. 7 / continuation 2

```
      2590                2610                2630
GGAAGCCCAGCCCAAGCACGGGGCTGGCAGGGCGTGAGGAACTCTCCTGTGGCCTGCTCA
      2650                2670                2690
TCACCCTTCCGACAGGAGCACTGCATGTCAGAGCACTTTAAAAACAGGCCAGCCTGCTTG
      2710                2730                2750
GGCCCTCGGTCTCCACCCCAGGGTCATAAGTGGGGAGAGAGCCCTTCCCAGGGCACCCAG
      2770                2790                2810
GCAGGTGCAGGGAAGTGCAGAGCTTGTGGAAAGCGTGTGAGTGAGGGAGACAGGAACGGC
      2830                2850                2870
TCTGGGGGTGGGAAGTGGGGCTAGGTCTTGCCAACTCCATCTTCAATAAAGTCGTTTTCG
      2890                2910
GATCCCTAAAAAAAAAAAAAAAAAAAAAAAAA
```

```
MGLSLPKEKGLILCLWSKFCRWFQRRESWAQSRDEQNLLQQKRIWESPLLLAAKDNDVQALNKLLKYEDCKVHQRGAMGETALHIA
ALYDNLEAAMVLMEAAPELVFEPMTSELYEGQTALHIAVVNQNMNLVRALLARRASVSARATGTAFRRSPRNLIYFGEHPLSFAAC
VNSEEIVRLLIEHGADIRAQDSLGNTVLHILILQPNKTFACQMYNLLLSYDRHGDHLQPLDLVPNHQGLTPFKLAGVEGNTVMFQH
LMQKRKHTQWTYGPLTSTLYDLTEIDSSGDEQSLLELIITTKKREARQILDQTPVKELVSLKWKRYGRPYFCMLGAIYLLYIICFT
MCCIYRPLKPRTNNRTSPRDNTLLQQKLLQEAYVTPKDDIRLVGELVTVIGAIIILLVEVPDIFRMGVTRFFGQTILGGPFHVLII
TYAFMVLVTMVMRLISASGEVVPMSFALVLGWCNVMYFARGFQMLGPFTIMIQKMIFGDLMRFCWLMAVVILGFASAFYIIFQTED
PEELGHFYDYPMALFSTFELFLTIIDGPANYNVDLPFMYSITYAAFAIIATLLMLNLLIAMMGDTHWRVAHERDELWRAQIVATTV
MLERKLPRCLWPRSGICGREYGLGDRWFLRVEDRQDLNRQRIQRYAQAFHTRGSEDLDKDSVEKLELGCPFSPHLSLPTPSVSRST
SRSSANWERLRQGTLRRDLRGIINRGLEDGESWEYQI
```

Figure 8:

A)

```
                    ATGGGTTTGTCACTGCCCAAGGAGAAAGGGCTAATTCTCT
                    M  G  L  S  L  P  K  E  K  G  L  I  L  C
       250                  270                  290
GCCTATGGAGCAAGTTCTGCAGATGGTTCCAGAGACGGGAGTCCTGGGCCCAGAGCCGAG
  L  W  S  K  F  C  R  W  F  Q  R  R  E  S  W  A  Q  S  R  D
       310                  330                  350
ATGAGCAGAACCTGCTGCAGCAGAAGAGGATCTGGGAGTCTCCTCTCCTTCTAGCTGCCA
  E  Q  N  L  L  Q  Q  K  R  I  W  E  S  P  L  L  L  A  A  K
       370                  390                  410
AAGATAATGATGTCCAGGCCCTGAACAAGTTGCTCAAGTATGAGGATTGCAAGGTGCACC
  D  N  D  V  Q  A  L  N  K  L  L  K  Y  E  D  C  K  V  H  Q
       430                  450                  470
AGAGAGGAGCCATGGGGGAAACAGCGCTACACATAGCAGCCCTCTATGACAACCTGGAGG
  R  G  A  M  G  E  T  A  L  H  I  A  A  L  Y  D  N  L  E  A
       490                  510                  530
CCGCCATGGTGCTGATGGAGGCTGCCCCGGAGCTGGTCTTTGAGCCCATGACATCTGAGC
  A  M  V  L  M  E  A  A  P  E  L  V  F  E  P  M  T  S  E  L
       550                  570                  590
TCTATGAGGGTCAGACTGCACTGCACATCGCTGTTGTGAACCAGAACATGAACCTGGTGC
  Y  E  G  Q  T  A  L  H  I  A  V  V  N  Q  N  M  N  L  V  R
       610                  630                  650
GAGCCCTGCTTGCCCGCAGGGCCAGTGTCTCTGCCAGAGCCACAGGCACTGCCTTCCGCC
  A  L  L  A  R  R  A  S  V  S  A  R  A  T  G  T  A  F  R  R
       670                  690                  710
GTAGTCCCTGCAACCTCATCTACTTTGGGGAGCACCCTTTGTCCTTTGCTGCCTGTGTGA
  S  P  C  N  L  I  Y  F  G  E  H  P  L  S  F  A  A  C  V  N
```

Fig. 8 / continuation 1

```
      730                 750                 770
ACAGTGAGGAGATCGTGCGGCTGCTCATTGAGCATGGAGCTGACATCCGGGCCCAGGACT
  S  E  E  I  V  R  L  L  I  E  H  G  A  D  I  R  A  Q  D  S
      790                 810                 830
CCCTGGGAAACACAGTGTTACACATCCTCATCCTCCAGCCCAACAAAACCTTTGCCTGCC
  L  G  N  T  V  L  H  I  L  I  L  Q  P  N  K  T  F  A  C  Q
      850                 870                 890
AGATGTACAACCTGTTGCTGTCCTACGACAGACATGGGGACCACCTGCAGCCCCTGGACC
  M  Y  N  L  L  L  S  Y  D  R  H  G  D  H  L  Q  P  L  D  L
      910                 930                 950
TCGTGCCCAATCACCAGGGTCTCACCCCTTTCAAGCTGGCTGGAGTGGAGGGTAACACTG
  V  P  N  H  Q  G  L  T  P  F  K  L  A  G  V  E  G  N  T  V
      970                 990                1010
TGATGTTTCAGCACCTGATGCAGAAGCGGAAGCACACCCAGTGGACGTATGGACCACTGA
  M  F  Q  H  L  M  Q  K  R  K  H  T  Q  W  T  Y  G  P  L  T
     1030                1050                1070
CCTCGACTCTCTATGACCTCACAGAGATCGACTCCTCAGGGGATGAGCAGTCCCTGCTGG
  S  T  L  Y  D  L  T  E  I  D  S  S  G  D  E  Q  S  L  L  E
     1090                1110                1130
AACTTATCATCACCACCAAGAAGCGGGAGGCTCGCCAGATCCTGGACCAGACGCCGGTGA
  L  I  I  T  T  K  K  R  E  A  R  Q  I  L  D  Q  T  P  V  K
     1150                1170                1190
AGGAGCTGGTGAGCCTCAAGTGGAAGCGGTACGGGCGGCCGTACTTCTGCATGCTGGGTG
  E  L  V  S  L  K  W  K  R  Y  G  R  P  Y  F  C  M  L  G  A
     1210                1230                1250
CCATATATCTGCTGTACATCATCTGCTTCACCATGTGCTGCATCTACCGCCCCCTCAAGC
  I  Y  L  L  Y  I  I  C  F  T  M  C  C  I  Y  R  P  L  K  P
     1270                1290                1310
CCAGGACCAATAACCGCACGAGCCCCCGGGACAACACCCTCTTACAGCAGAAGCTACTTC
  R  T  N  N  R  T  S  P  R  D  N  T  L  L  Q  Q  K  L  L  Q
     1330                1350                1370
AGGAAGCCTACATGACCCCTAAGGACGATATCCGGCTGGTCGGGGAGCTGGTGACTGTCA
  E  A  Y  M  T  P  K  D  D  I  R  L  V  G  E  L  V  T  V  I
     1390                1410                1430
TTGGGGCTATCATCATCCTGCTGGTAGAGGTTCCAGACATCTTCAGAATGGGGGTCACTC
  G  A  I  I  I  L  L  V  E  V  P  D  I  F  R  M  G  V  T  R
     1450                1470                1490
GCTTCTTTGGACAGACCATCCTTGGGGGCCCATTCCATGTCCTCATCATCACCTATGCCT
  F  F  G  Q  T  I  L  G  G  P  F  H  V  L  I  I  T  Y  A  F
     1510                1530                1550
TCATGGTGCTGGTGACCATGGTGATGCGGCTCATCAGTGCCAGCGGGGAGGTGGTACCCA
  M  V  L  V  T  M  V  M  R  L  I  S  A  S  G  E  V  V  P  M
     1570                1590                1610
TGTCCTTTGCACTCGTGCTGGGCTGGTGCAACGTCATGTACTTCGCCCGAGGATTCCAGA
  S  F  A  L  V  L  G  W  C  N  V  M  Y  F  A  R  G  F  Q  M
     1630                1650                1670
TGCTAGGCCCCTTCACCATCATGATTCAGAAGATGATTTTTGGCGACCTGATGCGATTCT
  L  G  P  F  T  I  M  I  Q  K  M  I  F  G  D  L  M  R  F  C
     1690                1710                1730
GCTGGCTGATGGCTGTGGTCATCCTGGGCTTTGCTTCAGCCTTCTATATCATCTTCCAGA
  W  L  M  A  V  V  I  L  G  F  A  S  A  F  Y  I  I  F  Q  T
     1750                1770                1790
CAGAGGACCCCGAGGAGCTAGGCCACTTCTACGACTACCCCATGGCCCTGTTCAGCACCT
  E  D  P  E  E  L  G  H  F  Y  D  Y  P  M  A  L  F  S  T  F
     1810                1830                1850
TCGAGCTGTTCCTTACCATCATCGATGGCCCAGCCAACTACAACGTGGACCTGCCCTTCA
  E  L  F  L  T  I  I  D  G  P  A  N  Y  N  V  D  L  P  F  M
     1870                1890                1910
TGTACAGCATCACCTATGCTGCCTTTGCCATCATCGCCACACTGCTCATGCTCAACCTCC
  Y  S  I  T  Y  A  A  F  A  I  I  A  T  L  L  M  L  N  L  L
     1930                1950                1970
TCATTGCCATGATGGGCGACACTCACTGGCGAGTGGCCCATGAGCGGGATGAGCTGTGGA
```

Fig. 8 / continu on 2

```
  I  A  M  M  G  D  T  H  W  R  V  A  H  E  R  D  E  L  W  R
     1990              2010              2030
GGGCCCAGATTGTGGCCACCACGGTGATGCTGGAGCGGAAGCTGCCTCGCTGCCTGTGGC
  A  Q  I  V  A  T  T  V  M  L  E  R  K  L  P  R  C  L  W  P
     2050              2070              2090
CTCGCTCCGGGATCTGCGGACGGGAGTATGGCCTGGGAGACCGCTGGTTCCTGCGGGTGG
  R  S  G  I  C  G  R  E  Y  G  L  G  D  R  W  F  L  R  V  E
     2110              2130              2150
AAGACAGGCAAGATCTCAACCGGCAGCGGATCCAACGCTACGCACAGGCCTTCCACACCC
  D  R  Q  D  L  N  R  Q  R  I  Q  R  Y  A  Q  A  F  H  T  R
     2170              2190              2210
GGGGCTCTGAGGATTTGGACAAAGACTCAGTGGAAAAACTAGAGCTGGGCTGTCCCTTCA
  G  S  E  D  L  D  K  D  S  V  E  K  L  E  L  G  C  P  F  S
     2230              2250              2270
GCCCCCACCTGTCCCTTCCTATGCCCTCAGTGTCTCGAAGTACCTCCCGCAGCAGTGCCA
  P  H  L  S  L  P  M  P  S  V  S  R  S  T  S  R  S  S  A  N
     2290              2310              2330
ATTGGGAAAGGCTTCGGCAAGGGACCCTGAGGAGAGACCTGCGTGGGATAATCAACAGGG
  W  E  R  L  R  Q  G  T  L  R  R  D  L  R  G  I  I  N  R  G
     2350              2370              2390
GTCTGGAGGACGGGGAGAGCTGGGAATATCAGATCTGA
  L  E  D  G  E  S  W  E  Y  Q  I  *
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MGLSLPKEKGLILCLWSKFCRWFQRRESWAQSRDEQNLLQQKRIWESPLLLAAKDNDVQALNKLLKYEDCKVHQRGAMGETALHIA
ALYDNLEAAMVLMEAAPELVFEPMTSELYEGQTALHIAVVNQNMNLVRALLARRASVSARATGTAFRRSPCNLIYFGEHPLSFAAC
VNSEEIVRLLIEHGADIRAQDSLGNTVLHILILQPNKTFACQMYNLLLSYDRHGDHLQPLDLVPNHQGLTPFKLAGVEGNTVMFQH
LMQKRKHTQWTYGPLTSTLYDLTEIDSSGDEQSLLELIITTKKREARQILDQTPVKELVSLKWKRYGRPYFCMLGAIYLLYIICFT
MCCIYRPLKPRTNNRTSPRDNTLLQQKLLQEAYMTPKDDIRLVGELVTVIGAIIILLVEVPDIFRMGVTRFFGQTILGGPFHVLII
TYAFMVLVTMVMRLISASGEVVPMSFALVLGWCNVMYFARGFQMLGPFTIMIQKMIFGDLMRFCWLMAVVILGFASAFYIIFQTED
PEELGHFYDYPMALFSTFELFLTIIDGPANYNVDLPFMYSITYAAFAIIATLLMLNLLIAMMGDTHWRVAHERDELWRAQIVATTV
MLERKLPRCLWPRSGICGREYGLGDRWFLRVEDRQDLNRQRIQRYAQAFHTRGSEDLDKDSVEKLELGCPFSPHLSLPMPSVSRST
SRSSANWERLRQGTLRRDLRGIINRGLEDGESWEYQI
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B)

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CAAACTCACAGCCCTCTCCAAACTGGCTGGGGCTGCTGGGAGACTCCCAAGGAACTCGTCAGGAAGGCAGGAGACAGGAGACGGGA
CCTCTACAGGGAGACGGTGGGCCGGCCCTTGGGGGGGCTGATGTGGCCCCAAGGCTGAGTCCCGTCAGGGTCTGGCCTCGGCCTCA
GGCCCCCAAGGAGCCGGCCCTACACCCCATGGGTTTGTCACTGCCCAAGGAGAAAGGGCTAATTCTCTGCCTATGGAGCAAGTTCT
GCAGATGGTTCCAGAGACGGGAGTCCTGGGCCCAGAGCCGAGATGAGCAGAACCTGCTGCAGCAGAAGAGGATCTGGGAGTCTCCT
CTCCTTCTAGCTGCCAAAGATAATGATGTCCAGGCCCTGAACAAGTTGCTCAAGTATGAGGATTGCAAGGTGCACCAGAGAGGAGC
CATGGGGGAAACAGCGCTACACATAGCAGCCCTCTATGACAACCTGGAGGCCGCCATGGTGCTGATGGAGGCTGCCCCGGAGCTGG
TCTTTGAGCCCATGACATCTGAGCTCTATGAGGGTCAGACTGCACTGCACATCGCTGTTGTGAACCAGAACATGAACCTGGTGCGA
GCCCTGCTTGCCCGCAGGGCCAGTGTCTCTGCCAGAGCCACAGGCACTGCCTTCCGCCGTAGTCCCCGCAACCTCATCTACTTTGG
GGAGCACCCTTTGTCCTTTGCTGCCTGTGTGAACAGTGAGGAGATCGTGCGGCTGCTCATTGAGCATGGAGCTGACATCCGGGCCC
AGGACTGCCTGGCCCAACAAAACCTTTGCCTGCCAGATGTACAACCTGTTGCTGTCCTACGACAGACATGGGGACCACCTGCAGCC
CCTGGACCTCGTGCCCAATCACCAGGGTCTCACCCCTTTCAAGCTGGCTGGAGTGGAGGGTAACACTGTGATGTTTCAGCACCTGA
TGCAGAAGCGGAAGCACACCCAGTGGACGTATGGACCACTGACCTCGACTCTCTATGACCTCACAGAGATCGACTCCTCAGGGGAT
GAGCAGTCCCTGCTGGAACTTATCATCACCACCAAGAAGCGGGAGGCTCGCCAGATCCTGGACCAGACGCCGGTGAAGGAGCTGGT
GAGCCTCAAGTGGAAGCGGTACGGGCGGCCGTACTTCTGCATGCTGGGTGCCATATATCTGCTGTACATCATCTGCTTCACCATGT
GCTGCATCTACCGCCCCCTCAAGCCCAGGACCAATAACCGCACAAGCCCCCGGGACAACACCCTCTTACAGCAGAAGCTACTTCAG
GAAGCCTACGTGACCCCTAAGGACGATATCCGGCTGGTCGGGGAGCTGGTGACTGTCATTGGGGCTATCATCATCCTGCTGGTAGA
GGTTCCAGACATCTTCAGAATGGGGGTCACTCGCTTCTTTGGACAGACCATCCTTGGGGGCCCATTCCATGTCCTCATCATCACCT
ATGCCTTCATGGTGCTGGTGACCATGGTGATGCGGCTCATATGATTTTGGCGACCTGATGCGATTCTGCTGGCTGATGGCTGTGG
TCATCCTGGGCTTTGCTTCAGCCTTCTATATCATCTTCCAGACAGAGGACCCCGAGGAGCTAGGCCACTTCTACGACTACCCCATG
GCCCTGTTCAGCACCTTCGAGCTGGTCCTTACCATCATCGATGGCCCAGCCAACTACAACGTGGACCTGCCCTTCATGTACAGCAT
CACCTATGCTGCCTTTGCCATCATCGCCACACTGCTCATGCTCAACCTCCTCATTGCCATGATGGGCGACACTCACTGGCGAGTGG
CCCATGAGCGGGATGAGCTGTGGAGGGCCCAGATTGTGGCCACCACGGTGATGCTGGAGCGGAAGCTGCCTCGCTGCCTGTGGCCT
CGCTCCGGGATCTGCGGACGGGAGTATGGCCTGGGGGACCGCTGGTTCCTGCGGGTGGAAGACAGGCAAGATCTCAACCGGCAGCG
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GATCCAACGCTACGCACAGGCCTTCCACACCCGGGGCTCTGAGGATTTGGACAAAGACTCAGTGGAAAAACTAGAGCTGGGCTGTC
CCTTCAGCCCCCACCTGTCCCTTCCTACGCCCTCAGTGTCTCGAAGTACCTCCCGCAGCAGTGCCAATTGGGAAAGGCTTCGGCAA
GGGACCCTGAGGAGAGACCTGCGTGGGATAATCAACAGGGGTCTGGAGGACGGGGAGAGCTGGGAATATCAGATCTGACTGCGTGT
TCTCACTTCGCTTCCTGGAACTTGCTCTCATTTTCCTGGGTGCATCAAACAAAACAAAAACCAAACACCCAGAGGTCTCATCTCCC
AGGCCCCAGGGAGAAAGAGGAGTAGCATGAACGCCAAGGAATGTACGTTGAGAATCACTGCTCCAGGCCTGCATTACTCCTTCAGC
TCTGGGGCAGAGGAAGCCCAGCCCAAGCACGGGGCTGGCAGGGCGTGAGGAACTCTCCTGTGGCCTGCTCATCACCCTTCCGACAG
GAGCACTGCATGTCAGAGCACTTTAAAAACAGGCCAGCCTGCTTGGGCCCTCGGTCTCCACCCCAGGGTCATAAGTGGGGAGAGAG
CCCTTCCCAGGGCACCCAGGCAGGTGCAGGGAAGTGCAGAGCTTGTGGAAAGCGTGTGAGTGAGGGAGACAGGAACGGCTCTGGGG
GTGGGAAGTGGGGCTAGGTCTTGCCAACTCCATCTTCAATAAAGTCGTTTTCGGATCCCTAAAAAAAAAAAAAAAAAAAAAAAAAA

c.)

CAAACTCACAGCCCTCTCCAAACTGGCTGGGGCTGCTGGGAGACTCCCAAGGAACTCGTCAGGAAGGCAGGAGACAGGAGACGGGA
CCTCTACAGGGAGACGGTGGGCCGGCCCTTGGGGGGGCTGATGTGGCCCCAAGGCTGAGTCCCGTCAGGGTCTGGCCTCGGCCTCA
GGCCCCCAAGGAGCCGGCCCTACACCCCATGGGTTTGTCACTGCCCAAGGAGAAAGGGCTAATTCTCTGCCTATGGAGCAAGTTCT
GCAGATGGTTCCAGAGACGGGAGTCCTGGGCCCAGAGCCGAGATGAGCAGAACCTGCTGCAGCAGAAGAGGATCTGGGAGTCTCCT
CTCCTTCTAGCTGCCAAAGATAATGATGTCCAGGCCCTGAACAAGTTGCTCAAGTATGAGGATTGCAAGGTGCACCAGAGAGGAGC
CATGGGGGAAACAGCGCTACACATAGCAGCCCTCTATGACAACCTGGAGGCCGCCATGGTGCTGATGGAGGCTGCCCCGGAGCTGG
TCTTTGAGCCCATGACATCTGAGCTCTATGAGGTCCTGACTGCCCATCACTTGAACGCCTGCCCCCTGAAATGCCAGGGCCTAGAG
AAGAGGAAGAGATGGGCAGCAGCTGGATCCCCTGGGAATCCTGAACACCCGAGAGCTCCCTGTTCTCCATCCCAGGCTACCCCTGA
GGGAAAGAGACTGGGGTGCATATGGGAGGGACCCCCTGCAGGATCCTGGGGACAGACCCGTGACTGACAGCTGTCTCTGGGCCAGG
TCAGACTGCACTGCACATCGCTGTTGTGAACCAGAACATGAACCTGGTGCGAGCCCTGCTTGCCCGCAGGGCCAGTGTCTCTGCCA
GAGCCACAGGCACTGCCTTCCGCCGTAGTCCCTGCAACCTCATCTACTTTGGGGAGCACCCTTTGTCCTTTGCTGCCTGTGTGAAC
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CCTTTCAAGCTGGCTGGAGTGGAGGGTAACACTGTGATGTTTCAGCACCTGATGCAGAAGCGGAAGCACACCCAGTGGACGTATGG
ACCACTGACCTCGACTCTCTATGACCTCACAGAGATCGACTCCTCAGGGGATGAGCAGTCCCTGCTGGAACTTATCATCACCACCA
AGAAGCGGGAGGCTCGCCAGATCCTGGACCAGACGCCGGTGAAGGAGCTGGTGAGCCTCAAGTGGAAGCGGTACGGGCGGCCGTAC
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TCCACACCCGGGGCTCTGAGGATTTGGACAAAGACTCAGTGGAAAAACTAGAGCTGGGCTGTCCCTTCAGCCCCCACCTGTCCCTT
CCTATGCCCTCAGTGTCTCGAAGTACCTCCCGCAGCAGTGCCAATTGGGAAAGGCTTCGGCAAGGGACCCTGAGGAGAGACCTGCG
TGGGATAATCAACAGGGGTCTGGAGGACGGGGAGAGCTGGGAATATCAGATCTGACTGCGTGTTCTCACTTCGCTTCCTGGAACTT
GCTCTCATTTTCCTGGGTGCATCAAACAAAACAAAAACCAAACACCCAGAGGTCTCATCTCCCAGGCCCCAGGGAGAAAGAGGAGT
AGCATGAACGCCAAGGAATGTACGTTGAGAATCACTGCTCCAGGCCTGCATTACTCCTTCAGCTCTGGGGCAGAGGAAGCCCAGCC
CAAGCACGGGGCTGGCAGGGCGTGAGGAACTCTCCTGTGGCCTGCTCATCACCCTTCCGACAGGAGCACTGCATGTCAGAGCACTT
TAAAAACAGGCCAGCCTGCTTGGGCCCTCGGTCTCCACCCCAGGGTCATAAGTGGGGAGAGAGCCCTTCCCAGGGCACCCAGGCAG
GTGCAGGGAAGTGCAGAGCTTGTGGAAAGCGTGTGAGTGAGGGAGACAGGAACGGCTCTGGGGGTGGGAAGTGGGGCTAGGTCTTG
CCAACTCCATCTTCAATAAAGTCGTTTTCGGATCCCTAAAAAAAAAAAAAAAAAAAAAAAAAA

D.)

CAAACTCACAGCCCTCTCCAAACTGGCTGGGGCTGCTGGGAGACTCCCAAGGAACTCGTCAGGAAGGCAGGAGACAGGAGACGGGA
CCTCTACAGGGAGACGGTGGGCCGGCCCTTGGGGGGGCTGATGTGGCCCCAAGGCTGAGTCCCGTCAGGGTCTGGCCTCGGCCTCA
GGCCCCCAAGGAGCCGGCCCTACACCCCATGGGTTTGTCACTGCCCAAGGAGAAAGGGCTAATTCTCTGCCTATGGAGCAAGTTCT
GCAGATGGTTCCAGAGACGGGAGTCCTGGGCCCAGAGCCGAGATGAGCAGAACCTGCTGCAGCAGAAGAGGATCTGGGAGTCTCCT
CTCCTTCTAGCTGCCAAAGATAATGATGTCCAGGCCCTGAACAAGTTGCTCAAGTATGAGGATTGCAAGGTGCACCAGAGAGGAGC
CATGGGGGAAACAGCGCTACACATAGCAGCCCTCTATGACAACCTGGAGGCCGCCATGGTGCTGATGGAGGCTGCCCCGGAGCTGG
TCTTTGAGCCCATGACATCTGAGCTCTATGAGGGTCAGACTGCACTGCACATCGCTGTTGTGAACCAGAACATGAACCTGGTGCGA
GCCCTGCTTGCCCGCAGGGCCAGTGTCTCTGCCAGAGCCACAGGCACTGCCTTCCGCCGTAGTCCCCGCAACCTCATCTACTTTGG

AAACACAGTGTTACACATCCTCATCCTCCAGCCCAACAAAACCTTTGCCTGCCAGATGTACAACCTGTTGCTGTCCTACGACAGAC
ATGGGGACCACCTGCAGCCCCTGGACCTCGTGCCCAATCACCAGGGTCTCACCCCTTTCAAGCTGGCTGGAGTGGAGGGTAACACT
GTGATGTTTCAGCACCTGATGCAGAAGCGGAAGCACACCCAGTGGACGTATGGACCACTGACCTCGACTCTCTATGACCTCACAGA
GATCGACTCCTCAGGGGATGAGCAGTCCCTGCTGGAACTTATCATCACCACCAAGAAGCGGGAGGCTCGCCAGATCCTGGACCAGA
CGCCGGTGAAGGAGCTGGTGAGCCTCAAGTGGAAGCGGTACGGGCGGCCGTACTTCTGCATGCTGGGTGCCATATATCTGCTGTAC
ATCATCTGCTTCACCATGTGCTGCATCTACCGCCCCCTCAAGCCCAGGACCAATAACCGCACAAGCCCCCGGGACAACACCCTCTT
ACAGCAGAAGCTACTTCAGGAAGCCTACGTGACCCCTAAGGACGATATCCGGCTGGTCGGGGAGCTGGTGACTGTCATTGGGGCTA
TCATCATCCTGCTGGTAGAGGTTCCAGACATCTTCAGAATGGGGGTCACTCGCTTCTTTGGACAGACCATCCTTGGGGGCCCATTC
CATGTCCTCATCATCACCTATGCCTTCATGGTGCTGGTGACCATGGTGATGCGGCTCATCAGTGCCAGCGGGGAGGTGGTACCCAT
GTCCTTTGCACTCGTGCTGGGCTGGTGCAACGTCATGTACTTCGCCCGAGGATTCCAGATGCTAGGCCCCTTCACCATCATGATTC
AGAAGATGATTTTTGGCGACCTGATGCGATTCTGCTGGCTGATGGCTGTGGTCATCCTGGGCTTTGCTTCAGCCTTCTATATCATC
TTCCAGACAGAGGACCCCGAGGAGCTAGGCCACTTCTACGACTACCCCATGGCCCTGTTCAGCACCTTCGAGCTGGTCCTTACCAT
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TCATGCTCAACCTCCTCATTGCCATGATGGGCGACACTCACTGGCGAGTGGCCCATGAGCGGGATGAGCTGTGGAGGGCCCAGATT
GTGGCCACCACGGTGATGCTGGAGCGGAAGCTGCCTCGCTGCCTGTGGCCTCGCTCCGGGATCTGCGGACGGGAGTATGGCCTGGG
GGACCGCTGGTTCCTGCGGGTGGAAGACAGGCAAGATCTCAACCGGCAGCGGATCCAACGCTACGCACAGGCCTTCCACACCCGGG
GCTCTGAGGATTTGGACAAAGACTCAGTGGAAAAACTAGAGCTGGGCTGTCCCTTCAGCCCCCACCTGTCCCTTCCTACGCCCTCA
GTGTCTCGAAGTACCTCCCGCAGCAGTGCCAATTGGGAAAGGCTTCGGCAAGGGACCCTGAGGAGAGACCTGCGTGGGATAATCAA
CAGGGGTCTGGAGGACGGGGAGAGCTGGGAATATCAGATCTGACTGCGTGTTCTCACTTCGCTTCCTGGAACTTGCTCTCATTTTC
CTGGGTGCATCAAACAAAACAAAAACCAAACACCCAGAGGTCTCATCTCCCAGGCCCCAGGGAGAAAGAGGAGTAGCATGAACGCC
AAGGAATGTACGTTGAGAATCACTGCTCCAGGCCTGCATTACTCCTTCAGCTCTGGGGCAGAGGAAGCCCAGCCCAAGCACGGGGC
TGGCAGGGCGTGAGGAACTCTCCTGTGGCCTGCTCATCACCCTTCCGACAGGAGCACTGCATGTCAGAGCACTTTAAAAACAGGCC
AGCCTGCTTGGGCCCTCGGTCTCCACCCCAGGGTCATAAGTGGGGAGAGAGCCCTTCCCAGGGCACCCAGGCAGGTGCAGGGAAGT
GCAGAGCTTGTGGAAAGCGTGTGAGTGAGGGAGACAGGAACGGCTCTGGGGGTGGGAAGTGGGGCTAGGTCTTGCCAACTCCATCT
TCAATAAAGTCGTTTTCGGATCCCTAAAAAAAAAAAAAAAAAAAAAAAA

E.)

CACACATGGGGCCTCCCAGGAGTGCCCAGGACCTCGTGCTGTTGGCCTCTGAATCTATCGTCTCCAATCCGCTGTCCCACAGAAGC
CATATAACCCACCTCTCTGTAAATGCCAGGAGCCATGGGGGAAACAGCGCTACACATAGCAGCCCTCTATGACAACCTGGAGGCCG
CCATGGTGCTGATGGAGGCTGCCCCGGAGCTGGTCTTTGAGCCCATGACATCTGAGCTCTATGGAGGGTGAGGGCCCACGGGTCTG
GGGTGAAGAGCAGGAGTGACGTGGTTGGGTATTCAAGTCAGTCTCTGTGATGGATAATTTGGGAAAGACACAGGGGATCTGAGCCT
CCTACTCTTTTTSTCTTCTCTGTCTCCCTTCCGTGTCAGTCCCTGACTGCCCATCACTTGAACGCCTGCCCCCTGAAATGCCAGGG
GCCTAGAGAAGAGGAAGAGATGGGCAGCAGCTGGATCCCCTGGGAATCCTGAACACCCGAGAGCTCCCTGTTCTCCATCCCAGGCT
ACCCCTGAGGGAAAGAGACTAGGGGTGCATATGGGAGGGACCCCCTGCAGGATCCTAGGGGACAGACCCGTGACTGACAGCTGTCT
CTGGGCCAGGTCAGACTGCACTGCACATCGCTGTTGTGAACCAGAACATGAACCTGGTGCGAGCCCTGCTTGCCCGCAGGGCCAGT
GTCTCTGCCAGAGCCACAGGCACTGCCTTCCGCCGTAGTCCCTGCAACCTCATCTACTTTGGGGAGCACCCTTTGTCCTTTGCTGC
CTGTGTGAACAGTGAGGAGATCGTGCGGCTGCTCATTGAGCATGGAGCTGACATCCGGGCCCAGGACTCCCTGGATGTACAACCTG
TTGCTGTCCTACGACAGACATGGGGACCACCTGCAGCCCCTGGACCTCGTGCCCAATCACCAGGGTCTCACCCCTTTCAAGCTGGC
TGGAGTGGAGGGTAACACTGTGATGTTTCAGCACCTGATGCAGAAGCGGAAGCACACCCAGTGGACGTATGGACCACTGACCTCGA
CTCTCTATGACCTCACAGAGATCGACTCCTCAGGGGATGAGCAGTCCCTGCTGGAACTTATCATCACCACCAAGAAGCGGGAGGCT
CGCCAGATCCTGGACCAGACGCCGGTGAAGGAGCTGGTGAGCCTCAAGTGGAAGCGGTACGGGCGGCCGTACTTCTGCATGCTGGG
TGCCATATATCTGCTGTACATCATCTGCTTCACCATGTGCTGCATCTACCGCCCCCTCAAGCCCAGGACCAATAACCGCACGAGCC
CCCGGGACAACACCCTCTTACAGCAGAAGCTACTTCAGGAAGCCTACATGACCCCTAAGGACGATATCCGGCTGGTCGGGGAGCTG
GTGACTGTCATTGGGGCTATCATCATCCTGCTGGTAGAGGTTCCAGACATCTTCAGAATGGGGGTCACTCGCTTCTTTGGACAGAC
CATCCTTGGGGGCCCATTCCATGTCCTCATCATCACCTATGCCTTCATGGTGCTGGTGACCATGGTGATGCGGCTCATCAGTGCCA
GCGGGGAGGTGGTACCCATGTCCTTTGCACTCGTGCTGGGCTGGTGCAACGTCATGTACTTCGCCCGAGGATTCCAGATGCTAGGC
CCCTTCACCATCATGATTCAGAAGATGATTTTTGGCGACCTGATGCGATTCTGCTGGCTGATGGCTGTGGTCATCCTGGGCTTTGC
TTCAGCCTTCTATATCATCTTCCAGACAGAGGACCCCGAGGAGCTAGGCCACTTCTACGACTACCCCATGGCCCTGTTCAGCACCT
TCGAGCTGGTCCTTACCATCATCGATGGCCCAGCCAACTACAACGTGGACCTGCCCTTCATGTACAGCATCACCTATGCTGCCTTT
GCCATCATCGCCACACTGCTCATGCTCAACCTCCTCATTGCCATGATGGGCGACACTCACTGGCGAGTGGCCCATGAGCGGGATGA
GCTGTGGAGGGCCCAGATTGTGGCCACCACGGTGATGCTGGAGCGGAAGCTGCCTCGCTGCCTGTGGCCTCGCTCCGGGATCTGCG
GACGGGAGTATGGCCTGGGAGACCGCTGGTTCCTGCGGGTGGAAGACAGGCAAGATCTCAACCGGCAGCGGATCCAACGCTACGCA
CAGGCCTTCCACACCCGGGGCTCTGAGGATTTGGACAAAGACTCAGTGGAAAAACTAGAGCTGGGCTGTCCCTTCAGCCCCCACCT
GTCCCTTCCTATGCCCTCAGTGTCTCGAAGTACCTCCCGCAGCAGTGCCAATTGGGAAAGGCTTCGGCAAGGGACCCTGAGGAGAG
ACCTGCGTGGGATAATCAACAGGGGTCTGGAGGACGGGGAGAGCTGGGAATATCAGATCTGACTGCGTGTTCTCACTTCGCTTCCT
GGAACTTGCTCTCATTTTCCTGGGTGCATCAAACAAAACAAAAACCAAACACCCAGAGGTCTCATCTCCCAGGCCCCAGGGAGAAA
GAGGAGTAGCATGAACGCCAAGGAATGTACGTTGAGAATCACTGCTCCAGGCCTGCATTACTCCTTCAGCTCTGGGGCAGAGGAAG
CCCAGCCCAAGCACGGGGCTGGCAGGGCGTGAGGAACTCTCCTGTGGCCTGCTCATCACCCTTCCGACAGGAGCACTGCATGTCAG
AGCACTTTAAAAACAGGCCAGCCTGCTTGGGCCCTCGGTCTCCACCCCAGGGTCATAAGTGGGGAGAGAGCCCTTCCCAGGGCACC

Fig. 8 / continuation 5

CAGGCAGGTGCAGGGAAGTGCAGAGCTTGTGGAAAGCGTGTGAGTGAGGGAGACAGGAACGGCTCTGGGGGTGGGAAGTGGGGCTA
GGTCTTGCCAACTCCATCTTCAATAAAGTCGTTTTCGGATCCCTAAAAAAAAAAAAAAAAAAAAAAAAAA

Figure 9:

A.

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        10                  30                  50
CGGGGCCCTGGGCTGCAGGAGGTTGCGGCGGCCGCGGCAGCATGGTGGTGCCGGAGAAGG
                                              M  V  V  P  E  K  E
        70                  90                 110
AGCAGAGCTGGATCCCCAAGATCTTCAAGAAGAAGACCTGCACGACGTTCATAGTTGACT
  Q  S  W  I  P  K  I  F  K  K  K  T  C  T  T  F  I  V  D  S
       130                 150                 170
CCACAGATCCGGGAGGGACCTTGTGCCAGTGTGGGCGCCCCCGGACCGCCCACCCCGCAG
  T  D  P  G  G  T  L  C  Q  C  G  R  P  R  T  A  H  P  A  V
       190                 210                 230
TGGCCATGGAGGATGCCTTCGGGGCAGCCGTGGTGACCGTGTGGGACAGCGATGCACACA
  A  M  E  D  A  F  G  A  A  V  V  T  V  W  D  S  D  A  H  T
       250                 270                 290
CCACGGAGAAGCCCACCGATGCCTACGGAGAGCTGGACTTCACGGGGGCCGGCCGCAAGC
  T  E  K  P  T  D  A  Y  G  E  L  D  F  T  G  A  G  R  K  H
       310                 330                 350
ACAGCAATTTCCTCCGGCTCTCTGACCGAACGGATCCAGCTGCAGTTTATAGTCTGGTCA
  S  N  F  L  R  L  S  D  R  T  D  P  A  A  V  Y  S  L  V  T
       370                 390                 410
CACGCACATGGGGCTTCCGTGCCCCGAACCTGGTGGTGTCAGTGCTGGGGGGATCGGGGG
  R  T  W  G  F  R  A  P  N  L  V  V  S  V  L  G  G  S  G  G
       430                 450                 470
GCCCCGTCCTCCAGACCTGGCTGCAGGACCTGCTGCGTCGTGGGCTGGTGCGGGCTGCCC
  P  V  L  Q  T  W  L  Q  D  L  L  R  R  G  L  V  R  A  A  Q
       490                 510                 530
AGAGCACAGGAGCCTGGATTGTCACTGGGGGTCTGCACACGGGCATCGGCCGGCATGTTG
  S  T  G  A  W  I  V  T  G  G  L  H  T  G  I  G  R  H  V  G
       550                 570                 590
GTGTGGCTGTACGGGACCATCAGATGGCCAGCACTGGGGGCACCAAGGTGGTGGCCATGG
  V  A  V  R  D  H  Q  M  A  S  T  G  G  T  K  V  V  A  M  G
       610                 630                 650
GTGTGGCCCCCTGGGGTGTGGTCCGGAATAGAGACACCCTCATCAACCCCAAGGGCTCGT
  V  A  P  W  G  V  V  R  N  R  D  T  L  I  N  P  K  G  S  F
       670                 690                 710
TCCCTGCGAGGTACCGGTGGCGCGGTGACCCGGAGGACGGGGTCCAGTTTCCCCTGGACT
  P  A  R  Y  R  W  R  G  D  P  E  D  G  V  Q  F  P  L  D  Y
       730                 750                 770
ACAACTACTCGGCCTTCTTCCTGGTGGACGACGGCACACACGGCTGCCTGGGGGGCGAGA
  N  Y  S  A  F  F  L  V  D  D  G  T  H  G  C  L  G  G  E  N
       790                 810                 830
ACCGCTTCCGCTTGCGCCTGGAGTCCTACATCTCACAGCAGAAGACGGGCGTGGGAGGGA
  R  F  R  L  R  L  E  S  Y  I  S  Q  Q  K  T  G  V  G  G  T
       850                 870                 890
CTGGAATTGACATCCCTGTCCTGCTCCTCCTGATTGATGGTGATGAGAAGATGTTGACGC
  G  I  D  I  P  V  L  L  L  L  I  D  G  D  E  K  M  L  T  R
       910                 930                 950
GAATAGAGAACGCCACCCAGGCTCAGCTCCCATGTCTCCTCGTGGCTGGCTCAGGGGGAG
  I  E  N  A  T  Q  A  Q  L  P  C  L  L  V  A  G  S  G  G  A
       970                 990                1010
CTGCGGACTGCCTGGCGGAGACCCTGGAAGACACTCTGGCCCCAGGGAGTGGGGGAGCCA
  A  D  C  L  A  E  T  L  E  D  T  L  A  P  G  S  G  G  A  R
      1030                1050                1070
GGCAAGGCGAAGCCCGAGATCGAATCAGGCGTTTCTTTCCCAAAGGGGACCTTGAGGTCC
```

Fig. 9 / continu: n 1

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 Q  G  E  A  R  D  R  I  R  R  F  F  P  K  G  D  L  E  V  L
     1090                1110                1130
TGCAGGCCCAGGTGGAGAGGATTATGACCCGGAAGGAGCTCCTGACAGTCTATTCTTCTG
 Q  A  Q  V  E  R  I  M  T  R  K  E  L  L  T  V  Y  S  S  E
     1150                1170                1190
AGGATGGGTCTGAGGAATTCGAGACCATAGTTTTGAAGGCCCTTGTGAAGGCCTGTGGGA
 D  G  S  E  E  F  E  T  I  V  L  K  A  L  V  K  A  C  G  S
     1210                1230                1250
GCTCGGAGGCCTCAGCCTACCTGGATGAGCTGCGTTTGGCTGTGGCTTGGAACCGCGTGG
 S  E  A  S  A  Y  L  D  E  L  R  L  A  V  A  W  N  R  V  D
     1270                1290                1310
ACATTGCCCAGAGTGAACTCTTTCGGGGGGACATCCAATGGCGGTCCTTCCATCTCGAAG
 I  A  Q  S  E  L  F  R  G  D  I  Q  W  R  S  F  H  L  E  A
     1330                1350                1370
CTTCCCTCATGGACGCCCTGCTGAATGACCGGCCTGAGTTCGTGCGCTTGCTCATTTCCC
 S  L  M  D  A  L  L  N  D  R  P  E  F  V  R  L  L  I  S  H
     1390                1410                1430
ACGGCCTCAGCCTGGGCCACTTCCTGACCCCGATGCGCCTGGCCCAACTCTACAGCGCGG
 G  L  S  L  G  H  F  L  T  P  M  R  L  A  Q  L  Y  S  A  A
     1450                1470                1490
CGCCCTCCAACTCGCTCATCCGCAACCTTTTGGACCAGGCGTCCCACAGCGCAGGCACCA
 P  S  N  S  L  I  R  N  L  L  D  Q  A  S  H  S  A  G  T  K
     1510                1530                1550
AAGCCCCAGCCCTAAAAGGGGGAGCTGCGGAGCTCCGGCCCCCTGACGTGGGGCATGTGC
 A  P  A  L  K  G  G  A  A  E  L  R  P  P  D  V  G  H  V  L
     1570                1590                1610
TGAGGATGCTGCTGGGGAAGATGTGCGCGCCGAGGTACCCCTCCGGGGGCGCCTGGGACC
 R  M  L  L  G  K  M  C  A  P  R  Y  P  S  G  G  A  W  D  P
     1630                1650                1670
CTCACCCAGGCCAGGGCTTCGGGGAGAGCATGTATCTGCTCTCGGACAAGGCCACCTCGC
 H  P  G  Q  G  F  G  E  S  M  Y  L  L  S  D  K  A  T  S  P
     1690                1710                1730
CGCTCTCGCTGGATGCTGGCCTCGGGCAGGCCCCCTGGAGCGACCTGCTTCTTTGGGCAC
 L  S  L  D  A  G  L  G  Q  A  P  W  S  D  L  L  L  W  A  L
     1750                1770                1790
TGTTGCTGAACAGGGCACAGATGGCCATGTACTTCTGGGAGATGGGTTCCAATGCAGTTT
 L  L  N  R  A  Q  M  A  M  Y  F  W  E  M  G  S  N  A  V  S
     1810                1830                1850
CCTCAGCTCTTGGGGCCTGTTTGCTGCTCCGGGTGATGGCACGCCTGGAGCCTGACGCTG
 S  A  L  G  A  C  L  L  L  R  V  M  A  R  L  E  P  D  A  E
     1870                1890                1910
AGGAGGCAGCACGGAGGAAAGACCTGGCGTTCAAGTTTGAGGGGATGGGCGTTGACCTCT
 E  A  A  R  R  K  D  L  A  F  K  F  E  G  M  G  V  D  L  F
     1930                1950                1970
TTGGCGAGTGCTATCGCAGCAGTGAGGTGAGGGCTGCCCGCCTCCTCCTCCGTCGCTGCC
 G  E  C  Y  R  S  S  E  V  R  A  A  R  L  L  L  R  R  C  P
     1990                2010                2030
CGCTCTGGGGGGATGCCACTTGCCTCCAGCTGGCCATGCAAGCTGACGCCCGTGCCTTCT
 L  W  G  D  A  T  C  L  Q  L  A  M  Q  A  D  A  R  A  F  F
     2050                2070                2090
TTGCCCAGGATGGGGTACAGTCTCTGCTGACACAGAAGTGGTGGGGAGATATGGCCAGCA
 A  Q  D  G  V  Q  S  L  L  T  Q  K  W  W  G  D  M  A  S  T
     2110                2130                2150
CTACACCCATCTGGGCCCTGGTTCTCGCCTTCTTTTGCCCTCCACTCATCTACACCCGCC
 T  P  I  W  A  L  V  L  A  F  F  C  P  P  L  I  Y  T  R  L
     2170                2190                2210
TCATCACCTTCAGGAAATCAGAAGAGGAGCCCACACGGGAGGAGCTAGAGTTTGACATGG
 I  T  F  R  K  S  E  E  E  P  T  R  E  E  L  E  F  D  M  D
     2230                2250                2270
ATAGTGTCATTAATGGGGAAGGGCCTGTCGGGACGGCGGACCCAGCCGAGAAGACGCCGC
 S  V  I  N  G  E  G  P  V  G  T  A  D  P  A  E  K  T  P  L
     2290                2310                2330
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Fig. 9 / continuation 2

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TGGGGGTCCCGCGCCAGTCGGGCCGTCCGGGTTGCTGCGGGGGCCGCTGCGGGGGGCGCC
  G  V  P  R  Q  S  G  R  P  G  C  C  G  G  R  C  G  G  R  R
      2350                2370                2390
GGTGCCTACGCCGCTGGTTCCACTTCTGGGGCGTGCCGGTGACCATCTTCATGGGCAACG
  C  L  R  R  W  F  H  F  W  G  V  P  V  T  I  F  M  G  N  V
      2410                2430                2450
TGGTCAGCTACCTGCTGTTCCTGCTGCTTTTCTCGCGGGTGCTGCTCGTGGATTTCCAGC
  V  S  Y  L  L  F  L  L  L  F  S  R  V  L  L  V  D  F  Q  P
      2470                2490                2510
CGGCGCCGCCCGGCTCCCTGGAGCTGCTGCTCTATTTCTGGGCTTTCACGCTGCTGTGCG
  A  P  P  G  S  L  E  L  L  L  Y  F  W  A  F  T  L  L  C  E
      2530                2550                2570
AGGAACTGCGCCAGGGCCTGAGCGGAGGCGGGGGCAGCCTCGCCAGCGGGGGCCCCGGGC
  E  L  R  Q  G  L  S  G  G  G  G  S  L  A  S  G  G  P  G  P
      2590                2610                2630
CTGGCCATGCCTCACTGAGCCAGCGCCTGCGCCTCTACCTCGCCGACAGCTGGAACCAGT
  G  H  A  S  L  S  Q  R  L  R  L  Y  L  A  D  S  W  N  Q  C
      2650                2670                2690
GCGACCTAGTGGCTCTCACCTGCTTCCTCCTGGGCGTGGGCTGCCGGCTGACCCCGGGTT
  D  L  V  A  L  T  C  F  L  L  G  V  G  C  R  L  T  P  G  L
      2710                2730                2750
TGTACCACCTGGGCCGCACTGTCCTCTGCATCGACTTCATGGTTTTCACGGTGCGGCTGC
  Y  H  L  G  R  T  V  L  C  I  D  F  M  V  F  T  V  R  L  L
      2770                2790                2810
TTCACATCTTCACGGTCAACAAACAGCTGGGGCCCAAGATCGTCATCGTGAGCAAGATGA
  H  I  F  T  V  N  K  Q  L  G  P  K  I  V  I  V  S  K  M  M
      2830                2850                2870
TGAAGGACGTGTTCTTCTTCCTCTTCTTCCTCGGCGTGTGGCTGGTAGCCTATGGCGTGG
  K  D  V  F  F  F  L  F  F  L  G  V  W  L  V  A  Y  G  V  A
      2890                2910                2930
CCACGGAGGGGCTCCTGAGGCCACGGGACAGTGACTTCCCAAGTATCCTGCGCCGCGTCT
  T  E  G  L  L  R  P  R  D  S  D  F  P  S  I  L  R  R  V  F
      2950                2970                2990
TCTACCGTCCCTACCTGCAGATCTTCGGGCAGATTCCCCAGGAGGACATGGACGTGGCCC
  Y  R  P  Y  L  Q  I  F  G  Q  I  P  Q  E  D  M  D  V  A  L
      3010                3030                3050
TCATGGAGCACAGCAACTGCTCGTCGGAGCCCGGCTTCTGGGCACACCCTCCTGGGGCCC
  M  E  H  S  N  C  S  S  E  P  G  F  W  A  H  P  P  G  A  Q
      3070                3090                3110
AGGCGGGCACCTGCGTCTCCCAGTATGCCAACTGGCTGGTGGTGCTGCTCCTCGTCATCT
  A  G  T  C  V  S  Q  Y  A  N  W  L  V  V  L  L  L  V  I  F
      3130                3150                3170
TCCTGCTCGTGGCCAACATCCTGCTGGTCAACTTGCTCATTGCCATGTTCAGTTACACAT
  L  L  V  A  N  I  L  L  V  N  L  L  I  A  M  F  S  Y  T  F
      3190                3210                3230
TCGGCAAAGTACAGGGCAACAGCGATCTCTACTGGAAGGCGCAGCGTTACCGCCTCATCC
  G  K  V  Q  G  N  S  D  L  Y  W  K  A  Q  R  Y  R  L  I  R
      3250                3270                3290
GGGAATTCCACTCTCGGCCCGCGCTGGCCCCGCCCTTTATCGTCATCTCCCACTTGCGCC
  E  F  H  S  R  P  A  L  A  P  P  F  I  V  I  S  H  L  R  L
      3310                3330                3350
TCCTGCTCAGGCAATTGTGCAGGCGACCCCGGAGCCCCCAGCCGTCCTCCCCGGCCCTCG
  L  L  R  Q  L  C  R  R  P  R  S  P  Q  P  S  S  P  A  L  E
      3370                3390                3410
AGCATTTCCGGGTTTACCTTTCTAAGGAAGCCGAGCGGAAGCTGCTAACGTGGGAATCGG
  H  F  R  V  Y  L  S  K  E  A  E  R  K  L  L  T  W  E  S  V
      3430                3450                3470
TGCATAAGGAGAACTTTCTGCTGGCACGCGCTAGGGACAAGCGGGAGAGCGACTCCGAGC
  H  K  E  N  F  L  L  A  R  A  R  D  K  R  E  S  D  S  E  R
      3490                3510                3530
GTCTGAAGCGCACGTCCCAGAAGGTGGACTTGGCACTGAAACAGCTGGGACACATCCGCG
  L  K  R  T  S  Q  K  V  D  L  A  L  K  Q  L  G  H  I  R  E
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Fig. 9 / continua ı 3

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      3550                3570                3590
AGTACGAACAGCGCCTGAAAGTGCTGGAGCGGGAGGTCCAGCAGTGTAGCCGCGTCCTGG
  Y  E  Q  R  L  K  V  L  E  R  E  V  Q  Q  C  S  R  V  L  G
      3610                3630                3650
GGTGGGTGGCCGAGGCCCTGAGCCGCTCTGCCTTGCTGCCCCCAGGTGGGCCGCCACCCC
  W  V  A  E  A  L  S  R  S  A  L  L  P  P  G  G  P  P  P  P
      3670                3690                3710
CTGACCTGCCTGGGTCCAAAGACTGAGCCCTGCTGGCGGACTTCAAGGAGAAGCCCCCAC
  D  L  P  G  S  K  D  *
      3730                3750                3770
AGGGGATTTTGCTCCTAGAGTAAGGCTCATCTGGGCCTCGGCCCCCGCACCTGGTGGCCT
      3790                3810                3830
TGTCCTTGAGGTGAGCCCCATGTCCATCTGGGCCACTGTCAGGACCACCTTTGGGAGTGT
      3850                3870                3890
CATCCTTACAAACCACAGCATGCCCGGCTCCTCCCAGAACCAGTCCCAGCCTGGGAGGAT
      3910                3930                3950
CAAGGCCTGGATCCCGGGCCGTTATCCATCTGGAGGCTGCAGGGTCCTTGGGGTAACAGG
      3970                3990                4010
GACCACAGACCCCTCACCACTCACAGATTCCTCACACTGGGGAAATAAAGCCATTTCAGA
      4030
GGAAAAAAAAAAAAAAAAAAAA
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MVVPEKEQSWIPKIFKKKTCTTFIVDSTDPGGTLCQCGRPRTAHPAVAMEDAFGAAVVTVWDSDAHTTEKPTDAYELDFTGAGRKH
SNFLRLSDRTDPAAVYSLVTRTWGFRAPNLVVSVLGGSGGPVLQTWLQDLLRRGLVRAAQSTGAWIVTGGLHTGIGRHVGVAVRDH
QMASTGGTKVVAMGVAPWGVVRNRDTLINPKGSFPARYRWRGDPEDGVQFPLDYNYSAFFLVDDGTHGCLGGENRFRLRLESYISQ
QKTGVGGTGIDIPVLLLLIDGDEKMLTRIENATQAHVPCLLVAGSRGLGMPGGTLEAHLAQDGDHKANQSTNQLLLPKDLSLQPVE
SIDRKTLQSYSERLAVAWNRVDIAQSELFRGDIQWRSFHLEASLMDALLNDRPEFVRLLISHGLSLGHFLTPMRLAQLYSAAPSNS
LIRNLLDQASHSAGTKAPALKGGAAELRPPDVGHVLRMLLGKMCAPRYPSGGAWDPHPGQGFGESMYLLSDKATSPLSLDAGLGQA
PWSDLLLWALLLNRAQMAMYFWEMGSNAVSSALGACLLLRVMARLEPDAEEAARRKDLAFKFEGMGVDLFGECYRSSEVRAARLLL
RRCPLWGDATCLQLAMQADARAFFAQDGVQSLLTQKWWGDMASTTPIWALVLAFFCPPLIYTRLITFRKSEEEPTREELEFDMDSV
INGEGPVGTADPAEKTPLGVPRQSGRPGCCGGRCGGRRCLRRWFHFWGVPVTIFMGNVVSYLLFLLLFSRVLLVDFQPAPPGSLEL
LLYFWAFTLLCEELRQGLSGGGGSLASGGPGPGHASLSQRLRLYLADSWNQCDLVALTCFLLGVGCRLTPGLYHLGRTVLCIDFMV
FTVRLLHIFTVNKQLGPKIVIVSKMMKDVFFFLFFLGVWLVAYGVATEGLLRPRDSDFPSILRRVFYRPYLQIFGQIPQEDMDVAL
MEHSNCSSEPGFWAHPPGAQAGTCVSQYANWLVVLLLVIFLLVANILLVNLLIAMFSYTFGKVQGNSDLYWKAQRYRLIREFHSRP
ALAPPFIVISHLRLLLRQLCRRPRSPQPSSPALEHFRVYLSKEAERKLLTWESVHKENFLLARARDKRESDSERLKRTSQKVDLAL
KQLGHIREYEQRLKVLEREVQQCSRVLGWVAEALSRSALLPPGGPPPPDLPGSKD
```

B.)

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       10                  30                  50
ATCCAATGGCGGTCCTTCCATCTCGAAGCTTCCCTCATGGACGCCCTGCTGAATGACCGG
       70                  90                  110
CCTGAGTTCGTGCGCTTGCTCATTTCCCACGGCCTCAGCCTGGGCCACTTCCTGACCCCG
       130                 150                 170
ATGCGCCTGGCCCAACTCTACAGCGCGGCGCCCTCCAACTCGCTCATCCGCAACCTTTTG
       190                 210                 230
GACCAGGCGTCCCACAGCGCAGGCACCAAAGCCCCAGCCCTAAAAGGGGGAGCTGCGGAG
       250                 270                 290
CTCCGGCCCCCTGACGTGGGGCATGTGCTGAGGATGCTGCTGGGGAAGATGTGCGCGCCG
       310                 330                 350
AGATGTATCTGCTCTCGGACAAGGCCACCTCGCCGCTCTCGCTGGATGCTGGCCTCGGGC
  M  Y  L  L  S  D  K  A  T  S  P  L  S  L  D  A  G  L  G  Q
       370                 390                 410
AGGCCCCCTGGAGCGACCTGCTTCTTTGGGCACTGTTGCTGAACAGGGCACAGATGGCCA
  A  P  W  S  D  L  L  L  W  A  L  L  L  N  R  A  Q  M  A  M
       430                 450                 470
TGTACTTCTGGGAGATGGGTTCCAATGCAGTTTCCTCAGCTCTTGGGGCCTGTTTGCTGC
  Y  F  W  E  M  G  S  N  A  V  S  S  A  L  G  A  C  L  L  L
```

Fig. 9 / continue 1 4

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      490                 510                 530
TCCGGGTGATGGCACGCCTGGAGCCTGACGCTGAGGAGGCAGCACGGAGGAAAGACCTGG
  R  V  M  A  R  L  E  P  D  A  E  E  A  A  R  R  K  D  L  A
      550                 570                 590
CGTTCAAGTTTGAGGGGATGGGCGTTGACCTCTTTGGCGAGTGCTATCGCAGCAGTGAGG
  F  K  F  E  G  M  G  V  D  L  F  G  E  C  Y  R  S  S  E  V
      610                 630                 650
TGAGGGCTGCCCGCCTCCTCCTCCGTCGCTGCCCGCTCTGGGGGGATGCCACTTGCCTCC
  R  A  A  R  L  L  L  R  R  C  P  L  W  G  D  A  T  C  L  Q
      670                 690                 710
AGCTGGCCATGCAAGCTGACGCCCGTGCCTTCTTTGCCCAGGATGGGGTACAGTCTCTGC
  L  A  M  Q  A  D  A  R  A  F  F  A  Q  D  G  V  Q  S  L  L
      730                 750                 770
TGACACAGAAGTGGTGGGGAGATATGGCCAGCACTACACCCATCTGGGCCCTGGTTCTCG
  T  Q  K  W  W  G  D  M  A  S  T  T  P  I  W  A  L  V  L  A
      790                 810                 830
CCTTCTTTTGCCCTCCACTCATCTACACCCGCCTCATCACCTTCAGGAAATCAGAAGAGG
  F  F  C  P  P  L  I  Y  T  R  L  I  T  F  R  K  S  E  E  E
      850                 870                 890
AGCCCACACGGGAGGAGCTAGAGTTTGACATGGATAGTGTCATTAATGGGGAAGGGCCTG
  P  T  R  E  E  L  E  F  D  M  D  S  V  I  N  G  E  G  P  V
      910                 930                 950
TCGGGACGGCGGACCCAGCCGAGAAGACGCCGCTGGGGGTCCCGCGCCAGTCGGGCCGTC
  G  T  A  D  P  A  E  K  T  P  L  G  V  P  R  Q  S  G  R  P
      970                 990                1010
CGGGTTGCTGCGGGGGCCGCTGCGGGGGGCGCCGGTGCCTACGCCGCTGGTTCCACTTCT
  G  C  C  G  G  R  C  G  G  R  R  C  L  R  R  W  F  H  F  W
     1030                1050                1070
GGGGCGTGCCGGTGACCATCTTCATGGGCAACGTGGTCAGCTACCTGCTGTTCCTGCTGC
  G  V  P  V  T  I  F  M  G  N  V  V  S  Y  L  L  F  L  L  L
     1090                1110                1130
TTTTCTCGCGGGTGCTGCTCGTGGATTTCCAGCCGGCGCCGCCCGGCTCCCTGGAGCTGC
  F  S  R  V  L  L  V  D  F  Q  P  A  P  P  G  S  L  E  L  L
     1150                1170                1190
TGCTCTATTTCTGGGCTTTCACGCTGCTGTGCGAGGAACTGCGCCAGGGCCTGAGCGGAG
  L  Y  F  W  A  F  T  L  L  C  E  E  L  R  Q  G  L  S  G  G
     1210                1230                1250
GCGGGGGCAGCCTCGCCAGCGGGGGCCCCGGGCCTGGCCATGCCTCACTGAGCCAGCGCC
  G  G  S  L  A  S  G  G  P  G  P  G  H  A  S  L  S  Q  R  L
     1270                1290                1310
TGCGCCTCTACCTCGCCGACAGCTGGAACCAGTGCGACCTAGTGGCTCTCACCTGCTTCC
  R  L  Y  L  A  D  S  W  N  Q  C  D  L  V  A  L  T  C  F  L
     1330                1350                1370
TCCTGGGCGTGGGCTGCCGGCTGACCCCGGGTTTGTACCACCTGGGCCGCACTGTCCTCT
  L  G  V  G  C  R  L  T  P  G  L  Y  H  L  G  R  T  V  L  C
     1390                1410                1430
GCATCGACTTCATGGTTTTCACGGTGCGGCTGCTTCACATCTTCACGGTCAACAAACAGC
  I  D  F  M  V  F  T  V  R  L  L  H  I  F  T  V  N  K  Q  L
     1450                1470                1490
TGGGGCCCAAGATCGTCATCGTGAGCAAGATGATGAAGGACGTGTTCTTCTTCCTCTTCT
  G  P  K  I  V  I  V  S  K  M  M  K  D  V  F  F  F  L  F  F
     1510                1530                1550
TCCTCGGCGTGTGGCTGGTAGCCTATGGCGTGGCCACGGAGGGGCTCCTGAGGCCACGGG
  L  G  V  W  L  V  A  Y  G  V  A  T  E  G  L  L  R  P  R  D
     1570                1590                1610
ACAGTGACTTCCCAAGTATCCTGCGCCGCGTCTTCTACCGTCCCTACCTGCAGATCTTCG
  S  D  F  P  S  I  L  R  R  V  F  Y  R  P  Y  L  Q  I  F  G
     1630                1650                1670
GGCAGATTCCCCAGGAGGACATGGACGTGGCCCTCATGGAGCACAGCAACTGCTCGTCGG
  Q  I  P  Q  E  D  M  D  V  A  L  M  E  H  S  N  C  S  S  E
     1690                1710                1730
AGCCCGGCTTCTGGGCACACCCTCCTGGGGCCCAGGCGGGCACCTGCGTCTCCCAGTATG
```

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```
  P  G  F  W  A  H  P  P  G  A  Q  A  G  T  C  V  S  Q  Y  A
     1750                 1770                 1790
CCAACTGGCTGGTGGTGCTGCTCCTCGTCATCTTCCTGCTCGTGGCCAACATCCTGCTGG
  N  W  L  V  V  L  L  L  V  I  F  L  L  V  A  N  I  L  L  V
     1810                 1830                 1850
TCAACTTGCTCATTGCCATGTTCAGTTACACATTCGGCAAAGTACAGGGCAACAGCGATC
  N  L  L  I  A  M  F  S  Y  T  F  G  K  V  Q  G  N  S  D  L
     1870                 1890                 1910
TCTACTGGAAGGCGCAGCGTTACCGCCTCATCCGGGAATTCCACTCTCGGCCCGCGCTGG
  Y  W  K  A  Q  R  Y  R  L  I  R  E  F  H  S  R  P  A  L  A
     1930                 1950                 1970
CCCCGCCCTTTATCGTCATCTCCCACTTGCGCCTCCTGCTCAGGCAATTGTGCAGGCGAC
  P  P  F  I  V  I  S  H  L  R  L  L  L  R  Q  L  C  R  R  P
     1990                 2010                 2030
CCCGGAGCCCCCAGCCGTCCTCCCCGGCCCTCGAGCATTTCCGGGTTTACCTTTCTAAGG
  R  S  P  Q  P  S  S  P  A  L  E  H  F  R  V  Y  L  S  K  E
     2050                 2070                 2090
AAGCCGAGCGGAAGCTGCTAACGTGGGAATCGGTGCATAAGGAGAACTTTCTGCTGGCAC
  A  E  R  K  L  L  T  W  E  S  V  H  K  E  N  F  L  L  A  R
     2110                 2130                 2150
GCGCTAGGGACAAGCGGGAGAGCGACTCCGAGCGTCTGAAGCGCACGTCCCAGAAGGTGG
  A  R  D  K  R  E  S  D  S  E  R  L  K  R  T  S  Q  K  V  D
     2170                 2190                 2210
ACTTGGCACTGAAACAGCTGGGACACATCCGCGAGTACGAACAGCGCCTGAAAGTGCTGG
  L  A  L  K  Q  L  G  H  I  R  E  Y  E  Q  R  L  K  V  L  E
     2230                 2250                 2270
AGCGGGAGGTCCAGCAGTGTAGCCGCGTCCTGGGGTGGGTGGCCGAGGCCCTGAGCCGCT
  R  E  V  Q  Q  C  S  R  V  L  G  W  V  A  E  A  L  S  R  S
     2290                 2310                 2330
CTGCCTTGCTGCCCCCAGGTGGGCCGCCACCCCCTGACCTGCCTGGGTCCAAAGACTGAG
  A  L  L  P  P  G  G  P  P  P  P  D  L  P  G  S  K  D  *
     2350                 2370                 2390
CCCTGCTGGCGGACTTCAAGGAGAAGCCCCCACAGGGGATTTTGCTCCTAGAGTAAGGCT
     2410                 2430                 2450
CATCTGGGCCTCGGCCCCCGCACCTGGTGGCCTTGTCCTTGAGGTGAGCCCCATGTCCAT
     2470                 2490                 2510
CTGGGCCACTGTCAGGACCACCTTTGGGAGTGTCATCCTTACAAACCACAGCATGCCCGG
     2530                 2550                 2570
CTCCTCCCAGAACCAGTCCCAGCCTGGGAGGATCAAGGCCTGGATCCCGGGCCGTTATCC
     2590                 2610                 2630
ATCTGGAGGCTGCAGGGTCCTTGGGGTAACAGGGACCACAGACCCCTCACCACTCACAGA
     2650                 2670                 2690
TTCCTCACACTGGGGAAATAAAGCCATTTCAGAGGAAAAAAAAAAAAAAAAAAAA
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MYLLSDKATSPLSLDAGLGQAPWSDLLLWALLLNRAQMAMYFWEMGSNAVSSALGACLLLRVMARLEPDAEEAARRKDLAFKFEGM
GVDLFGECYRSSEVRAARLLLRRCPLWGDATCLQLAMQADARAFFAQDGVQSLLTQKWWGDMASTTPIWALVLAFFCPPLIYTRLI
TFRKSEEEPTREELEFDMDSVINGEGPVGTADPAEKTPLGVPRQSGRPGCCGGRCGGRRCLRRWFHFWGVPVTIFMGNVVSYLLFL
LLFSRVLLVDFQPAPPGSLELLLYFWAFTLLCEELRQGLSGGGGSLASGGPGPGHASLSQRLRLYLADSWNQCDLVALTCFLLGVG
CRLTPGLYHLGRTVLCIDFMVFTVRLLHIFTVNKQLGPKIVIVSKMMKDVFFFLFFLGVWLVAYGVATEGLLRPRDSDFPSILRRV
FYRPYLQIFGQIPQEDMDVALMEHSNCSSEPGFWAHPPGAQAGTCVSQYANWLVVLLLVIFLLVANILLVNLLIAMFSYTFGKVQG
NSDLYWKAQRYRLIREFHSRPALAPPFIVISHLRLLLRQLCRRPRSPQPSSPALEHFRVYLSKEAERKLLTWESVHKENFLLARAR
DKRESDSERLKRTSQKVDLALKQLGHIREYEQRLKVLEREVQQCSRVLGWVAEALSRSALLPPGGPPPPDLPGSKD
```

A)

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        10                  30                  50
ATTAAAGTTTATAAAACAGTGGCTGGATGGTTGGAGGATGCAGGTGGACAGAAGACGTGG
                          M  V  G  G  C  R  W  T  E  D  V  E
        70                  90                  110
AGCCTGCAGAAGTAAAGGAAAAGATGTCCTTTCGGGCAGCCAGGCTCAGCATGAGGAACA
  P  A  E  V  K  E  K  M  S  F  R  A  A  R  L  S  M  R  N  R
       130                 150                 170
GAAGGAATGACACTCTGGACAGCACCCGGACCCTGTACTCCAGCGCGTCTCGGAGCACAG
  R  N  D  T  L  D  S  T  R  T  L  Y  S  S  A  S  R  S  T  D
       190                 210                 230
ACTTGTCTTACAGTGAAAGCGCCAGCTTCTACGCTGCCTTCAGGACACAGACGTGCCCAA
  L  S  Y  S  E  S  A  S  F  Y  A  A  F  R  T  Q  T  C  P  I
       250                 270                 290
TCATGGCTTCTTGGGACTTGGTGAATTTTATTCAAGCAAATTTTAAGAAACGAGAATGTG
  M  A  S  W  D  L  V  N  F  I  Q  A  N  F  K  K  R  E  C  V
       310                 330                 350
TCTTCTTTACCAAAGATTCCAAGGCCACGGAGAATGTGTGCAAGTGTGGCTATGCCCAGA
  F  F  T  K  D  S  K  A  T  E  N  V  C  K  C  G  Y  A  Q  S
       370                 390                 410
GCCAGCACATGGAAGGCACCCAGATCAACCAAAGTGAGAAATGGAACTACAAGAAACACA
  Q  H  M  E  G  T  Q  I  N  Q  S  E  K  W  N  Y  K  K  H  T
       430                 450                 470
CCAAGGAATTTCCTACCGACGCCTTTGGGGATATTCAGTTTGAGACACTGGGGAAGAAAG
  K  E  F  P  T  D  A  F  G  D  I  Q  F  E  T  L  G  K  K  G
       490                 510                 530
GGAAGTATATACGTCTGTCCTGCGACACGGACGCGGAAATCCTTTACGAGCTGCTGACCC
  K  Y  I  R  L  S  C  D  T  D  A  E  I  L  Y  E  L  L  T  Q
       550                 570                 590
AGCACTGGCACCTGAAAACACCCAACCTGGTCATTTCTGTGACCGGGGGCGCCAAGAACT
  H  W  H  L  K  T  P  N  L  V  I  S  V  T  G  G  A  K  N  F
       610                 630                 650
TCGCCCTGAAGCCGCGCATGCGCAAGATCTTCAGCCGGCTCATCTACATCGCGCAGTCCA
  A  L  K  P  R  M  R  K  I  F  S  R  L  I  Y  I  A  Q  S  K
       670                 690                 710
AAGGTGCTTGGATTCTCACGGGAGGCACCCATTATGGCCTGATGAAGTACATCGGGGAGG
  G  A  W  I  L  T  G  G  T  H  Y  G  L  M  K  Y  I  G  E  V
       730                 750                 770
TGGTGAGAGATAACACCATCAGCAGGAGTTCAGAGGAGAATATTGTGGCCATTGGCATAG
  V  R  D  N  T  I  S  R  S  S  E  E  N  I  V  A  I  G  I  A
       790                 810                 830
CAGCTTGGGGCATGGTCTCCAACCGGGACACCCTCATCAGGAATTGCGATGCTGAGGGCT
  A  W  G  M  V  S  N  R  D  T  L  I  R  N  C  D  A  E  G  Y
       850                 870                 890
ATTTTTTAGCCCAGTACCTTATGGATGACTTCACAAGAGATCCACTGTATATCCTGGACA
  F  L  A  Q  Y  L  M  D  D  F  T  R  D  P  L  Y  I  L  D  N
       910                 930                 950
ACAACCACACACATTTGCTGCTCGTGGACAATGGCTGTCATGGACATCCCACTGTCGAAG
  N  H  T  H  L  L  L  V  D  N  G  C  H  G  H  P  T  V  E  A
       970                 990                1010
CAAAGCTCCGGAATCAGCTAGAGAAGTATATCTCTGAGCGCACTATTCAAGATTCCAACT
  K  L  R  N  Q  L  E  K  Y  I  S  E  R  T  I  Q  D  S  N  Y
      1030                1050                1070
ATGGTGGCAAGATCCCCATTGTGTGTTTTGCCCAAGGAGGTGGAAAAGAGACTTTGAAAG
  G  G  K  I  P  I  V  C  F  A  Q  G  G  G  K  E  T  L  K  A
      1090                1110                1130
CCATCAATACCTCCATCAAAAATAAAATTCCTTGTGTGGTGGTGGAAGGCTCGGGCCAGA
  I  N  T  S  I  K  N  K  I  P  C  V  V  V  E  G  S  G  Q  I
      1150                1170                1190
TCGCTGATGTGATCGCTAGCCTGGTGGAGGTGGAGGATGCCCTGACATCTTCTGCCGTCA
  A  D  V  I  A  S  L  V  E  V  E  D  A  L  T  S  S  A  V  K
      1210                1230                1250
```

Fig. 10 / continuation 1

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AGGAGAAGCTGGTGCGCTTTTTACCCCGCACGGTGTCCCGGCTGCCTGAGGAGGAGACTG
  E  K  L  V  R  F  L  P  R  T  V  S  R  L  P  E  E  E  T  E
     1270                1290                1310
AGAGTTGGATCAAATGGCTCAAAGAAATTCTCGAATGTTCTCACCTATTAACAGTTATTA
  S  W  I  K  W  L  K  E  I  L  E  C  S  H  L  L  T  V  I  K
     1330                1350                1370
AAATGGAAGAAGCTGGGGATGAAATTGTGAGCAATGCCATCTCCTACGCTCTATACAAAG
  M  E  E  A  G  D  E  I  V  S  N  A  I  S  Y  A  L  Y  K  A
     1390                1410                1430
CCTTCAGCACCAGTGAGCAAGACAAGGATAACTGGAATGGGCAGCTGAAGCTTCTGCTGG
  F  S  T  S  E  Q  D  K  D  N  W  N  G  Q  L  K  L  L  L  E
     1450                1470                1490
AGTGGAACCAGCTGGACTTAGCCAATGATGAGATTTTCACCAATGACCGCCGATGGGAGA
  W  N  Q  L  D  L  A  N  D  E  I  F  T  N  D  R  R  W  E  K
     1510                1530                1550
AGAGCAAACCGAGGCTCAGAGACACAATAATCCAGGTCACATGGCTGGAAAATGGTAGAA
  S  K  P  R  L  R  D  T  I  I  Q  V  T  W  L  E  N  G  R  I
     1570                1590                1610
TCAAGGTTGAGAGCAAAGATGTGACTGACGGCAAAGCCTCTTCTCATATGCTGGTGGTTC
  K  V  E  S  K  D  V  T  D  G  K  A  S  S  H  M  L  V  V  L
     1630                1650                1670
TCAAGTCTGCTGACCTTCAAGAAGTCATGTTTACGGCTCTCATAAAGGACAGACCCAAGT
  K  S  A  D  L  Q  E  V  M  F  T  A  L  I  K  D  R  P  K  F
     1690                1710                1730
TTGTCCGCCTCTTTCTGGAGAATGGCTTGAACCTACGGAAGTTTCTCACCCATGATGTCC
  V  R  L  F  L  E  N  G  L  N  L  R  K  F  L  T  H  D  V  L
     1750                1770                1790
TCACTGAACTCTTCTCCAACCACTTCAGCACGCTTGTGTACCGGAATCTGCAGATCGCCA
  T  E  L  F  S  N  H  F  S  T  L  V  Y  R  N  L  Q  I  A  K
     1810                1830                1850
AGAATTCCTATAATGATGCCCTCCTCACGTTTGTCTGGAAACTGGTTGCGAACTTCCGAA
  N  S  Y  N  D  A  L  L  T  F  V  W  K  L  V  A  N  F  R  R
     1870                1890                1910
GAGGCTTCCGGAAGGAAGACAGAAATGGCCGGGACGAGATGGACATAGAACTCCACGACG
  G  F  R  K  E  D  R  N  G  R  D  E  M  D  I  E  L  H  D  V
     1930                1950                1970
TGTCTCCTATTACTCGGCACCCCCTGCAAGCTCTCTTCATCTGGGCCATTCTTCAGAATA
  S  F  I  T  R  H  P  L  Q  A  L  F  I  W  A  I  L  Q  N  K
     1990                2010                2030
AGAAGGAACTCTCCAAAGTCATTTGGGAGCAGACCAGGGGCTGCACTCTGGCAGCCCTGG
  K  E  L  S  K  V  I  W  E  Q  T  R  G  C  T  L  A  A  L  G
     2050                2070                2090
GAGCCAGCAAGCTTCTGAAGACTCTGGCCAAAGTGAAGAACGACATCAATGCTGCTGGGG
  A  S  K  L  L  K  T  L  A  K  V  K  N  D  I  N  A  A  G  E
     2110                2130                2150
AGTCCGAGGAGCTGGCTAATGAGTACGAGACCCGGGCTGTTGGTGAGTCCACAGTGTGGA
  S  E  E  L  A  N  E  Y  E  T  R  A  V  G  E  S  T  V  W  N
     2170                2190                2210
ATGCTGTGGTGGGCGCGGATCTGCCATGTGGCACAGACATTGCCAGCGGCACTCATAGAC
  A  V  V  G  A  D  L  P  C  G  T  D  I  A  S  G  T  H  R  P
     2230                2250                2270
CAGATGGTGGAGAGCTGTTCACTGAGTGTTACAGCAGCGATGAAGACTTGGCAGAACAGC
  D  G  G  E  L  F  T  E  C  Y  S  S  D  E  D  L  A  E  Q  L
     2290                2310                2330
TGCTGGTCTATTCCTGTGAAGCTTGGGGTGGAAGCAACTGTCTGGAGCTGGCGGTGGAGG
  L  V  Y  S  C  E  A  W  G  G  S  N  C  L  E  L  A  V  E  A
     2350                2370                2390
CCACAGACCAGCATTTCATCGCCCAGCCTGGGGTCCAGAATTTTCTTTCTAAGCAATGGT
  T  D  Q  H  F  I  A  Q  P  G  V  Q  N  F  L  S  K  Q  W  Y
     2410                2430                2450
ATGGAGAGATTTCCCGAGACACCAAGAACTGGAAGATTATCCTGTGTCTGTTTATTATAC
  G  E  I  S  R  D  T  K  N  W  K  I  I  L  C  L  F  I  I  P
```

Fig. 10 / continuation 2

```
     2470                2490                2510
CCTTGGTGGGCTGTGGCTTTGTATCATTTAGGAAGAAACCTGTCGACAAGCACAAGAAGC
  L  V  G  C  G  F  V  S  F  R  K  K  P  V  D  K  H  K  K  L
     2530                2550                2570
TGCTTTGGTACTATGTGGCGTTCTTCACCTCCCCCTTCGTGGTCTTCTCCTGGAATGTGG
  L  W  Y  Y  V  A  F  F  T  S  P  F  V  V  F  S  W  N  V  V
     2590                2610                2630
TCTTCTACATCGCCTTCCTCCTGCTGTTTGCCTACGTGCTGCTCATGGATTTCCATTCGG
  F  Y  I  A  F  L  L  L  F  A  Y  V  L  L  M  D  F  H  S  V
     2650                2670                2690
TGCCACACCCCCCCGAGCTGGTCCTGTACTCGCTGGTCTTTGTCCTCTTCTGTGATGAAG
  P  H  P  P  E  L  V  L  Y  S  L  V  F  V  L  F  C  D  E  V
     2710                2730                2750
TGAGACAGGGCCGGCCGGCTGCTCCCAGTGCGGGGCCCGCCAAGCCCACGCCCACCCGGA
  R  Q  G  R  P  A  A  P  S  A  G  P  A  K  P  T  P  T  R  N
     2770                2790                2810
ACTCCATCTGGCCCGCAAGCTCCACACGCAGCCCCGGTTCCCGCTCACGCCACTCCTTCC
  S  I  W  P  A  S  S  T  R  S  P  G  S  R  S  R  H  S  F  H
     2830                2850                2870
ACACTTCCCTGCAAGCTGAGGGTGCCAGCTCTGGCCTTGGCCAGCCCAGAAAGGGGTGGA
  T  S  L  Q  A  E  G  A  S  S  G  L  G  Q  P  R  K  G  W  T
     2890                2910                2930
CATTTAAAAATCTGGAAATGGTTGATATTTCCAAGCTGCTGATGTCCCTCTCTGTCCCTT
  F  K  N  L  E  M  V  D  I  S  K  L  L  M  S  L  S  V  P  F
     2950                2970                2990
TCTGTACGCAGTGGTACGTAAATGGGGTGAATTATTTTACTGACCTGTGGAATGTGATGG
  C  T  Q  W  Y  V  N  G  V  N  Y  F  T  D  L  W  N  V  M  D
     3010                3030                3050
ACACGCTGGGGCTTTTTTACTTCATAGCAGGAATTGTATTTCGGCAAGGGATCCTTAGGC
  T  L  G  L  F  Y  F  I  A  G  I  V  F  R  Q  G  I  L  R  Q
     3070                3090                3110
AGAATGAGCAGCGCTGGAGGTGGATATTCCGTTCGGTCATCTACGAGCCCTACCTGGCCA
  N  E  Q  R  W  R  W  I  F  R  S  V  I  Y  E  P  Y  L  A  M
     3130                3150                3170
TGTTCGGCCAGGTGCCCAGTGACGTGGATGGTACCACGTATGACTTTGCCCACTGCACCT
  F  G  Q  V  P  S  D  V  D  G  T  T  Y  D  F  A  H  C  T  F
     3190                3210                3230
TCACTGGGAATGAGTCCAAGCCACTGTGTGTGGAGCTGGATGAGCACAACCTGCCCCGGT
  T  G  N  E  S  K  P  L  C  V  E  L  D  E  H  N  L  P  R  F
     3250                3270                3290
TCCCCGAGTGGATCACCATCCCCCTGGTGTGCATCTACATGTTATCCACCAACATCCTGC
  P  E  W  I  T  I  P  L  V  C  I  Y  M  L  S  T  N  I  L  L
     3310                3330                3350
TGGTCAACCTGCTGGTCGCCATGTTTGGCTACACGGTGGGCACCGTCCAGGAGAACAATG
  V  N  L  L  V  A  M  F  G  Y  T  V  G  T  V  Q  E  N  N  D
     3370                3390                3410
ACCAGGTCTGGAAGTTCCAGAGGTACTTCCTGGTGCAGGAGTACTGCAGCCGCCTCAATA
  Q  V  W  K  F  Q  R  Y  F  L  V  Q  E  Y  C  S  R  L  N  I
     3430                3450                3470
TCCCCTTCCCCTTCATCGTCTTCGCTTACTTCTACATGGTGGTGAAGAAGTGCTTCAAGT
  P  F  P  F  I  V  F  A  Y  F  Y  M  V  V  K  K  C  F  K  C
     3490                3510                3530
GTTGCTGCAAGGAGAAAAACATGGAGTCTTCTGTCTGCTGTGAGTGGTTTATCCATGTGT
  C  C  K  E  K  N  M  E  S  S  V  C  C  E  W  F  I  H  V  Y
     3550                3570                3590
ACTTGGGATCAGAAGCAGCGATTAATTTCAGGGAAGGATGCCTGCATCCAGTGATTGGAA
  L  G  S  E  A  A  I  N  F  R  E  G  C  L  H  P  V  I  G  S
     3610                3630                3650
GCTGGACCCCAGGCTGGCTGGTCTGGACATCCACACGCATTCTCACATGCAGTGCCGGCT
  W  T  P  G  W  L  V  W  T  S  T  R  I  L  T  C  S  A  G  W
     3670                3690                3710
GGCCAGCAGCAGGGAGTCTCAGTGTCACCACACATAGCAGCTGGGTTCCTGCAAAAAGCA
```

Fig. 10 / continuation 3

```
  P  A  A  G  S  L  S  V  T  T  H  S  S  W  V  P  A  K  S  S
     3730                 3750                 3770
GCAAGTCACAGGCCCACCCAGACAGAACGGGTAGAGAATGTGACTCTGCTTCTGGGTGGG
  K  S  Q  A  H  P  D  R  T  G  R  E  C  D  S  A  S  G  W  E
     3790                 3810                 3830
AAGGACAGCCTGCCCGGTGGGTGGAAGAATCCGTGGCCCTGTTTGGCCATCGTGGCCCTG
  G  Q  P  A  R  W  V  E  E  S  V  A  L  F  G  H  R  G  P  V
     3850                 3870                 3890
TTTGGCCACCTACCACTCTAGGCATCACTGAGCTGAATGCGCCGGTCCTCTGA
  W  P  P  T  T  L  G  I  T  E  L  N  A  P  V  L  *
```

```
MVGGCRWTEDVEPAEVKEKMSFRAARLSMRNRRNDTLDSTRTLYSSASRSTDLSYSESASFYAAFRTQTCPIMASWDLVNFIQANF
KKRECVFFTKDSKATENVCKCGYAQSQHMEGTQINQSEKWNYKKHTKEFPTDAFGDIQFETLGKKGKYIRLSCDTDAEILYELLTQ
HWHLKTPNLVISVTGGAKNFALKPRMRKIFSRLIYIAQSKGAWILTGGTHYGLMKYIGEVVRDNTISRSSEENIVAIGIAAWGMVS
NRDTLIRNCDAEGYFLAQYLMDDFTRDPLYILDNNHTHLLLVDNGCHGHPTVEAKLRNQLEKYISERTIQDSNYGGKIPIVCFAQG
GGKETLKAINTSIKNKIPCVVVEGSGQIADVIASLVEVEDALTSSAVKEKLVRFLPRTVSRLPEEETESWIKWLKEILECSHLLTV
IKMEEAGDEIVSNAISYALYKAFSTSEQDKDNWNGQLKLLLEWNQLDLANDEIFTNDRRWEKSKPRLRDTIIQVTWLENGRIKVES
KDVTDGKASSHMLVVLKSADLQEVMFTALIKDRPKFVRLFLENGLNLRKFLTHDVLTELFSNHFSTLVYRNLQIAKNSYNDALLTF
VWKLVANFRRGFRKEDRNGRDEMDIELHDVSPITRHPLQALFIWAILQNKKELSKVIWEQTRGCTLAALGASKLLKTLAKVKNDIN
AAGESEELANEYETRAVGESTVWNAVVGADLPCGTDIASGTHRPDGGELFTECYSSDEDLAEQLLVYSCEAWGGSNCLELAVEATD
QHFIAQPGVQNFLSKQWYGEISRDTKNWKIILCLFIIPLVGCGFVSFRKKPVDKHKKLLWYYVAFFTSPFVVFSWNVVFYIAFLLL
FAYVLLMDFHSVPHPPELVLYSLVFVLFCDEVRQGRPAAPSAGPAKPTPTRNSIWPASSTRSPGSRSRHSFHTSLQAEGASSGLGQ
PRKGWTFKNLEMVDISKLLMSLSVPFCTQWYVNGVNYFTDLWNVMDTLGLFYFIAGIVFRQGILRQNEQRWRWIFRSVIYEPYLAM
FGQVPSDVDGTTYDFAHCTFTGNESKPLCVELDEHNLPRFPEWITIPLVCIYMLSTNILLVNLLVAMFGYTVGTVQENNDQVWKFQ
RYFLVQEYCSRLNIPFPFIVFAYFYMVVKKCFKCCCKEKNMESSVCCEWFIHVYLGSEAAINFREGCLHPVIGSWTPGWLVWTSTR
ILTCSAGWPAAGSLSVTTHSSWVPAKSSKSQAHPDRTGRECDSASGWEGQPARWVEESVALFGHRGPVWPPTTLGITELNAPVL
```

B.

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                                                        Q  L
     2290                 2310                 2330
TGCTGGTCTATTCCTGTGAAGCTTGGGGTGGAAGCAACTGTCTGGAGCTGGCGGTGGAGG
  L  V  Y  S  C  E  A  W  G  G  S  N  C  L  E  L  A  V  E  A
     2350                 2370                 2390
CCACAGACCAGCATTTCATCGCCCAGCCTGGGGTCCAGAATTTTCTTTCTAAGCAATGGT
  T  D  Q  H  F  I  A  Q  P  G  V  Q  N  F  L  S  K  Q  W  Y
     2410                 2430                 2450
ATGGAGAGATTTCCCGAGACACCAAGAACTGGAAGATTATCCTGTGTCTGTTTATTATAC
  G  E  I  S  R  D  T  K  N  W  K  I  I  L  C  L  F  I  I  P
     2470                 2490                 2510
CCTTGGTGGGCTGTGGCTTTGTATCATTTAGGAAGAAACCTGTCGACAAGCACAAGAAGC
  L  V  G  C  G  F  V  S  F  R  K  K  P  V  D  K
```

Figure 11:

a.) Trp10b cDNA and derived amino acid sequence

```
        10                  30                  50
ATGAAATCCTTCCTTCCTGTCCACACCATCGTGCTTATCAGGGAGAATGTGTGCAAGTGT
M  K  S  F  L  P  V  H  T  I  V  L  I  R  E  N  V  C  K  C
        70                  90                  110
GGCTATGCCCAGAGCCAGCACATGGAAGGCACCCAGATCAACCAAAGTGAGAAATGGAAC
G  Y  A  Q  S  Q  H  M  E  G  T  Q  I  N  Q  S  E  K  W  N
       130                 150                 170
TACAAGAAACACACCAAGGAATTTCCTACCGACGCCTTTGGGGATATTCAGTTTGAGACA
Y  K  K  H  T  K  E  F  P  T  D  A  F  G  D  I  Q  F  E  T
       190                 210                 230
CTGGGGAAGAAAGGGAAGTATATACGTCTGTCCTGCGACACGGACGCGGAAATCCTTTAC
L  G  K  K  G  K  Y  I  R  L  S  C  D  T  D  A  E  I  L  Y
       250                 270                 290
GAGCTGCTGACCCAGCACTGGCACCTGAAAACACCCAACCTGGTCATTTCTGTGACCGGG
E  L  L  T  Q  H  W  H  L  K  T  P  N  L  V  I  S  V  T  G
       310                 330                 350
GGCGCCAAGAACTTCGCCCTGAAGCCGCGCATGCGCAAGATCTTCAGCCGGCTCATCTAC
G  A  K  N  F  A  L  K  P  R  M  R  K  I  F  S  R  L  I  Y
       370                 390                 410
ATCGCGCAGTCCAAAGGTGCTTGGATTCTCACGGGAGGCACCCATTATGGCCTGATGAAG
I  A  Q  S  K  G  A  W  I  L  T  G  G  T  H  Y  G  L  M  K
       430                 450                 470
TACATCGGGGAGGTGGTGAGAGATAACACCATCAGCAGGAGTTCAGAGGAGAATATTGTG
Y  I  G  E  V  V  R  D  N  T  I  S  R  S  S  E  E  N  I  V
       490                 510                 530
GCCATTGGCATAGCAGCTTGGGGCATGGTCTCCAACCGGGACACCCTCATCAGGAATTGC
A  I  G  I  A  A  W  G  M  V  S  N  R  D  T  L  I  R  N  C
       550                 570                 590
GATGCTGAGGGCTATTTTTTAGCCCAGTACCTTATGGATGACTTCACAAGAGATCCACTG
D  A  E  G  Y  F  L  A  Q  Y  L  M  D  D  F  T  R  D  P  L
       610                 630                 650
TATATCCTGGACAACAACCACACACATTTGCTGCTCGTGGACAATGGCTGTCATGGACAT
Y  I  L  D  N  N  H  T  H  L  L  L  V  D  N  G  C  H  G  H
       670                 690                 710
CCCACTGTCGAAGCAAAGCTCCGGAATCAGCTAGAGAAGTATATCTCTGAGCGCACTATT
P  T  V  E  A  K  L  R  N  Q  L  E  K  Y  I  S  E  R  T  I
       730                 750                 770
CAAGATTCCAACTATGGTGGCAAGATCCCCATTGTGTGTTTTGCCCAAGGAGGTGGAAAA
Q  D  S  N  Y  G  G  K  I  P  I  V  C  F  A  Q  G  G  G  K
       790                 810                 830
GAGACTTTGAAAGCCATCAATACCTCCATCAAAAATAAAATTCCTTGTGTGGTGGTGGAA
E  T  L  K  A  I  N  T  S  I  K  N  K  I  P  C  V  V  V  E
       850                 870                 890
GGCTCGGGCCAGATCGCTGATGTGATCGCTAGCCTGGTGGAGGTGGAGGATGCCCTGACA
G  S  G  Q  I  A  D  V  I  A  S  L  V  E  V  E  D  A  L  T
       910                 930                 950
TCTTCTGCCGTCAAGGAGAAGCTGGTGCGCTTTTTACCCCGCACGGTGTCCCGGCTGCCT
S  S  A  V  K  E  K  L  V  R  F  L  P  R  T  V  S  R  L  P
       970                 990                1010
GAGGAGGAGACTGAGAGTTGGATCAAATGGCTCAAAGAAATTCTCGAATGTTCTCACCTA
E  E  E  T  E  S  W  I  K  W  L  K  E  I  L  E  C  S  H  L
      1030                1050                1070
TTAACAGTTATTAAAATGGAAGAAGCTGGGGATGAAATTGTGAGCAATGCCATCTCCTAC
L  T  V  I  K  M  E  E  A  G  D  E  I  V  S  N  A  I  S  Y
      1090                1110                1130
GCTCTATACAAAGCCTTCAGCACCAGTGAGCAAGACAAGGATAACTGGAATGGGCAGCTG
A  L  Y  K  A  F  S  T  S  E  Q  D  K  D  N  W  N  G  Q  L
```

Fig. 11 (Continuation)

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      2410                2430                2450
AGAAACTTAGGACCCAAGATTATAATGCTGCAGAGGATGCTGATCGATGTGTTCTTCTTC
R  N  L  G  P  K  I  I  M  L  Q  R  M  L  I  D  V  F  F  F
      2470                2490                2510
CTGTTCCTCTTTGCGGTGTGGATGGTGGCCTTTGGCGTGGCCAGGCAAGGGATCCTTAGG
L  F  L  F  A  V  W  M  V  A  F  G  V  A  R  Q  G  I  L  R
      2530                2550                2570
CAGAATGAGCAGCGCTGGAGGTGGATATTCCGTTCGGTCATCTACGAGCCCTACCTGGCC
Q  N  E  Q  R  W  R  W  I  F  R  S  V  I  Y  E  P  Y  L  A
      2590                2610                2630
ATGTTCGGCCAGGTGCCCAGTGACGTGGATGGTACCACGTATGACTTTGCCCACTGCACC
M  F  G  Q  V  P  S  D  V  D  G  T  T  Y  D  F  A  H  C  T
      2650                2670                2690
TTCACTGGGAATGAGTCCAAGCCACTGTGTGTGGAGCTGGATGAGCACAACCTGCCCCGG
F  T  G  N  E  S  K  P  L  C  V  E  L  D  E  H  N  L  P  R
      2710                2730                2750
TTCCCCGAGTGGATCACCATCCCCCTGGTGTGCATCTACATGTTATCCACCAACATCCTG
F  P  E  W  I  T  I  P  L  V  C  I  Y  M  L  S  T  N  I  L
      2770                2790                2810
CTGGTCAACCTGCTGGTCGCCATGTTTGGCTACACGGTGGGCACCGTCCAGGAGAACAAT
L  V  N  L  L  V  A  M  F  G  Y  T  V  G  T  V  Q  E  N  N
      2830                2850                2870
GACCAGGTCTGGAAGTTCCAGAGGTACTTCCTGGTGCAGGAGTACTGCAGCCGCCTCAAT
D  Q  V  W  K  F  Q  R  Y  F  L  V  Q  E  Y  C  S  R  L  N
      2890                2910                2930
ATCCCCTTCCCCTTCATCGTCTTCGCTTACTTCTACATGGTGGTGAAGAAGTGCTTCAAG
I  P  F  P  F  I  V  F  A  Y  F  Y  M  V  V  K  K  C  F  K
      2950                2970                2990
TGTTGCTGCAAGGAGAAAAACATGGAGTCTTCTGTCTGCTGTTTCAAAAATGAAGACAAT
C  C  C  K  E  K  N  M  E  S  S  V  C  C  F  K  N  E  D  N
      3010                3030                3050
GAGACTCTGGCATGGGAGGGTGTCATGAAGGAAAACTACCTTGTCAAGATCAACACAAAA
E  T  L  A  W  E  G  V  M  K  E  N  Y  L  V  K  I  N  T  K
      3070                3090                3110
GCCAACGACACCTCAGAGGAAATGAGGCATCGATTTAGACAACTGGATACAAAGCTTAAT
A  N  D  T  S  E  E  M  R  H  R  F  R  Q  L  D  T  K  L  N
      3130                3150
GATCTCAAGGGTCTACTGAAAGAGATTGCTAATAAAATCAAATAG
D  L  K  G  L  L  K  E  I  A  N  K  I  K  *
```

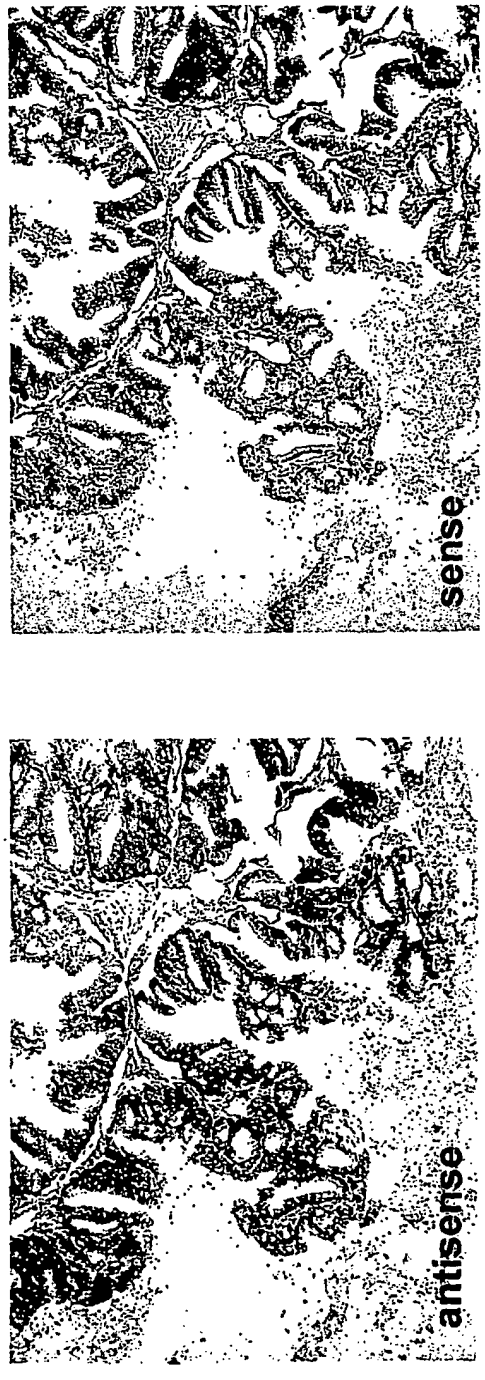
b.) Trp10 protein:

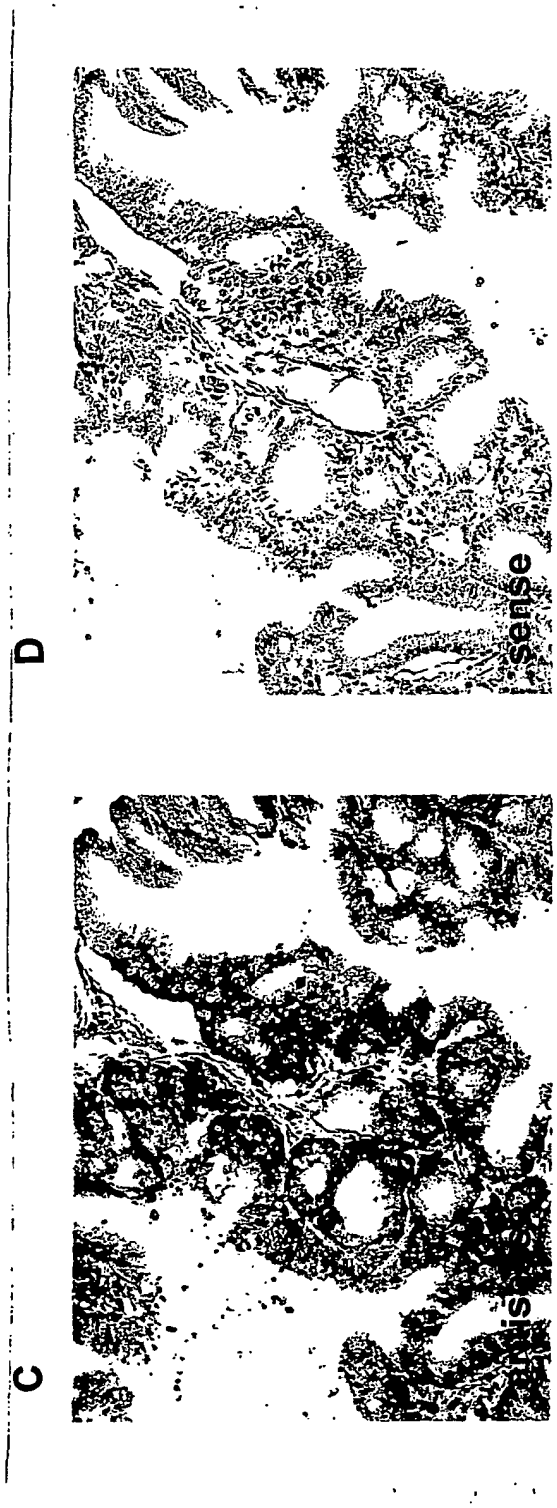
```
MKSFLPVHTIVLIRENVCKCGYAQSQHMEGTQINQSEKWNYKKHTKEFPTDAFGDIQFETLGKKGKYIRLSCDTDAEILY
ELLTQHWHLKTPNLVISVTGGAKNFALKPRMRKIFSRLIYIAQSKGAWILTGGTHYGLMKYIGEVVRDNTISRSSEENIV
AIGIAAWGMVSNRDTLIRNCDAEGYFLAQYLMDDFTRDPLYILDNNHTHLLLVDNGCHGHPTVEAKLRNQLEKYISERTI
QDSNYGGKIPIVCFAQGGGKETLKAINTSIKNKIPCVVVEGSGQIADVIASLVEVEDALTSSAVKEKLVRFLPRTVSRLP
EEETESWIKWLKEILECSHLLTVIKMEEAGDEIVSNAISYALYKAFSTSEQDKDNWNGQLKLLLEWNQLDLANDEIFTND
RRWESADLQEVMFTALIKDRPKFVRLFLENGLNLRKFLTHDVLTELFSNHFSTLVYRNLQIAKNSYNDALLTFVWKLVAN
FRRGFRKEDRNGRDEMDIELHDVSPITRHPLQALFIWAILQNKKELSKVIWEQTRGCTLAALGASKLLKTLAKVKNDINA
AGESEELANEYETRAVELFTECYSSDEDLAEQLLVYSCEAWGGSNCLELAVEATDQHFIAQPGVQNFLSKQWYGEISRDT
KNWKIILCLFIIPLVGCGFVSFRKKPVDKHKKLLWYYVAFFTSPFVVFSWNVVFYIAFLLLFAYVLLMDFHSVPHPPELV
LYSLVFVLFCDEVRQWYVNGVNYFTDLWNVMDTLGLFYFIAGIVFRLHSSNKSSLYSGRVIFCLDYIIFTLRLIHIFTVS
RNLGPKIIMLQRMLIDVFFFLFLFAVWMVAFGVARQGILRQNEQRWRWIFRSVIYEPYLAMFGQVPSDVDGTTYDFAHCT
FTGNESKPLCVELDEHNLPRFPEWITIPLVCIYMLSTNILLVNLLVAMFGYTVGTVQENNDQVWKFQRYFLVQEYCSRLN
IPFPFIVFAYFYMVVKKCFKCCCKEKNMESSVCCFKNEDNETLAWEGVMKENYLVKINTKANDTSEEMRHRFRQLDTKLN
DLKGLLKEIANKIK
```

Figs. 12A and 12B

The Trp8 gene is expressed in endometrial or uterine cancer, but not in normal endometrium

Endometrial cancer:



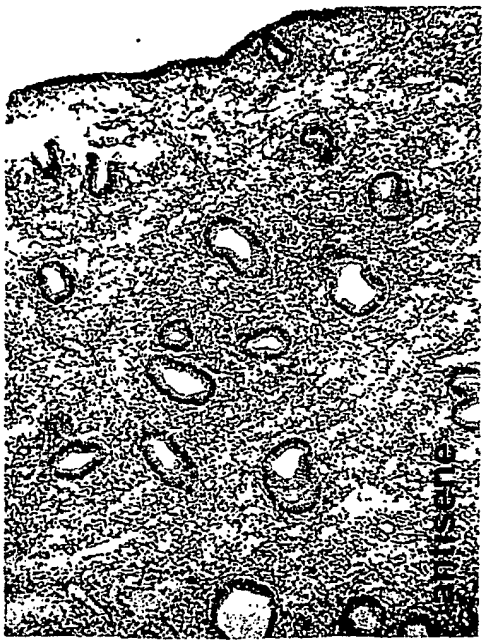


Figs. 12C and 12D

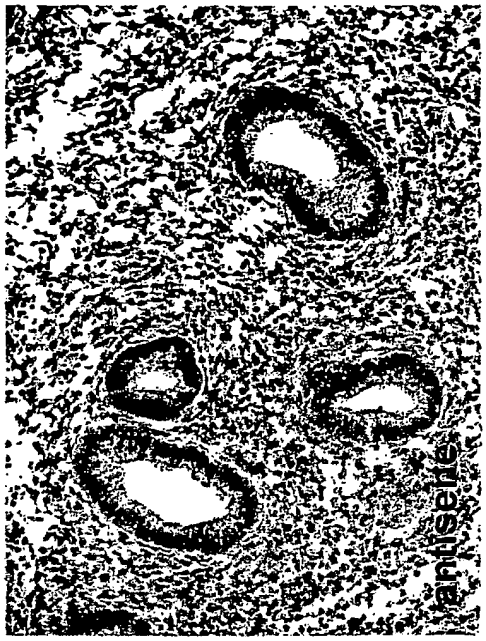
Figs. 12E and 12F

Endometrium:

E



F



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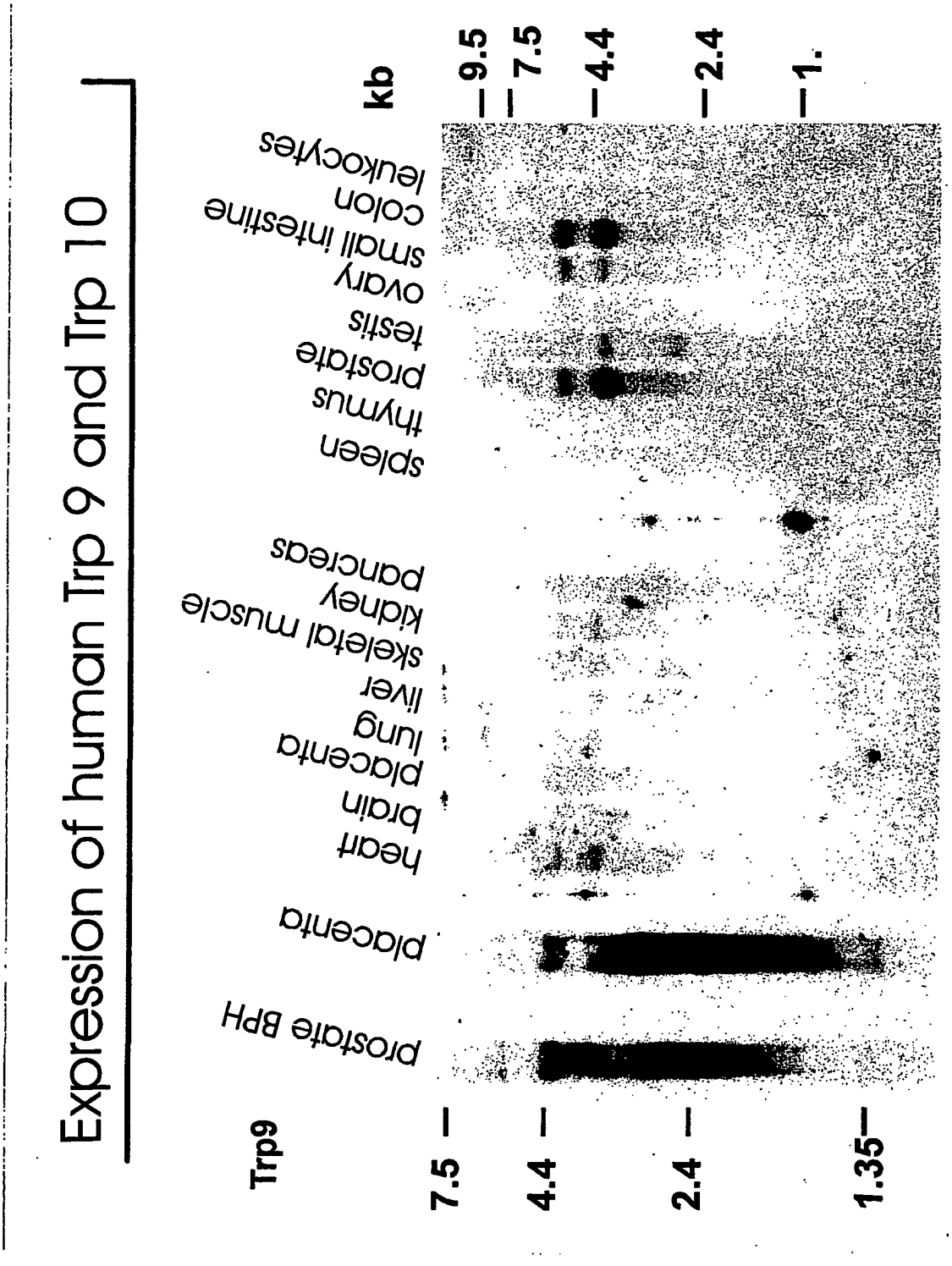


Fig. 13

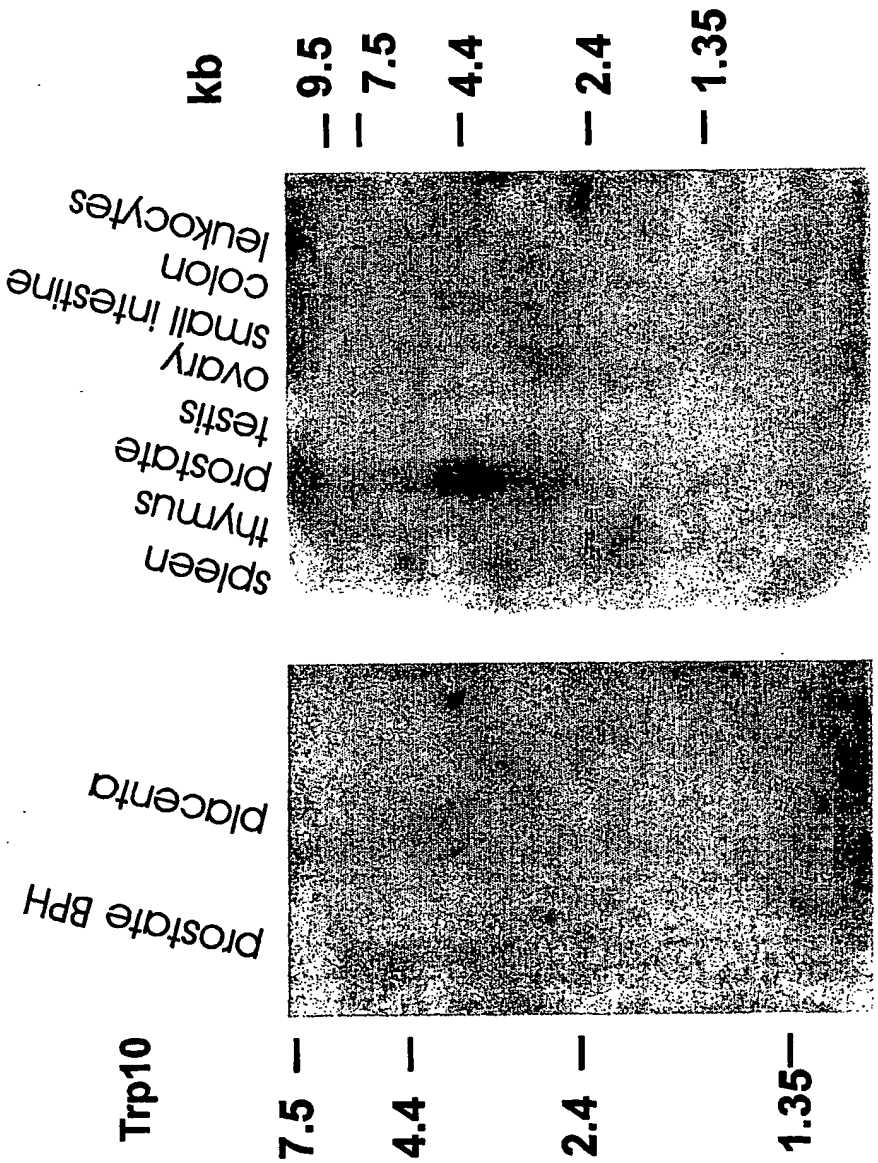
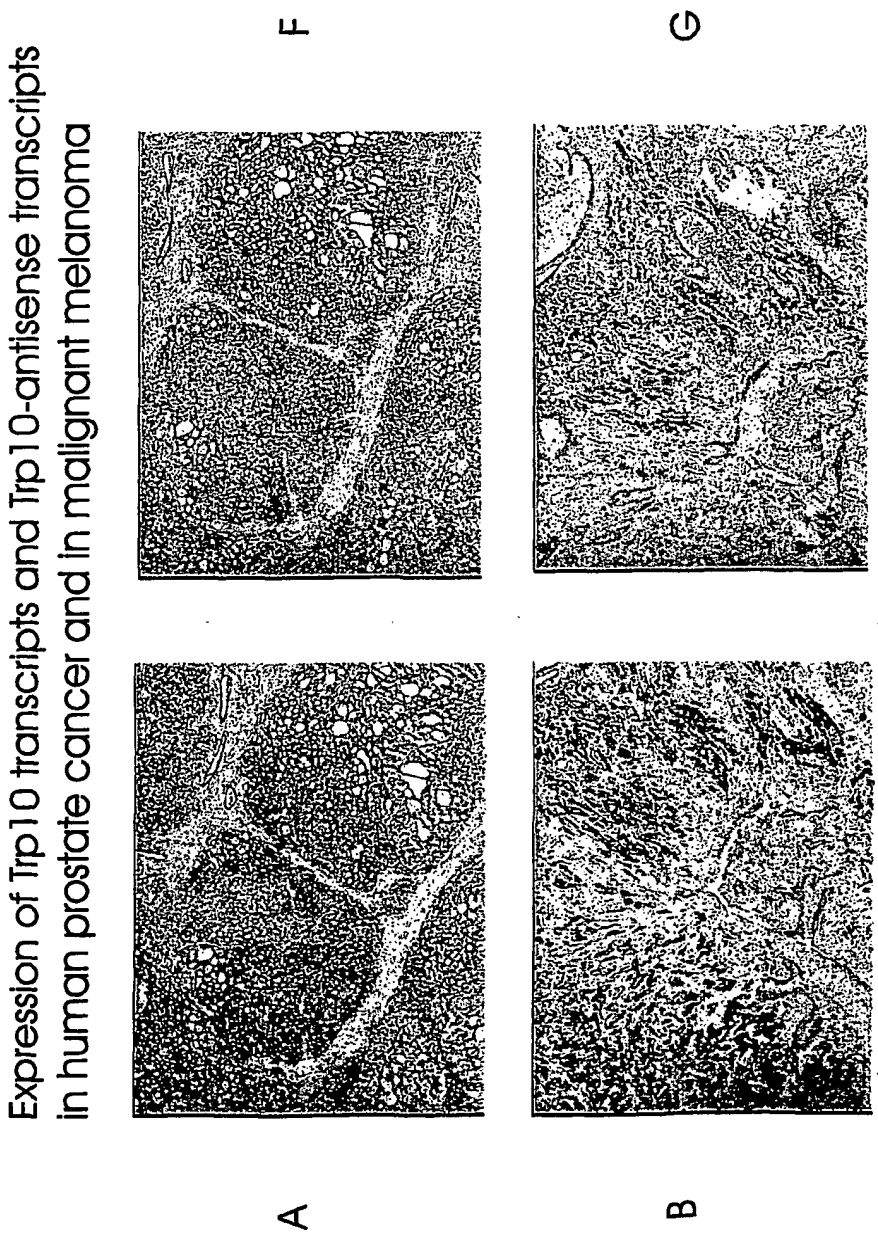
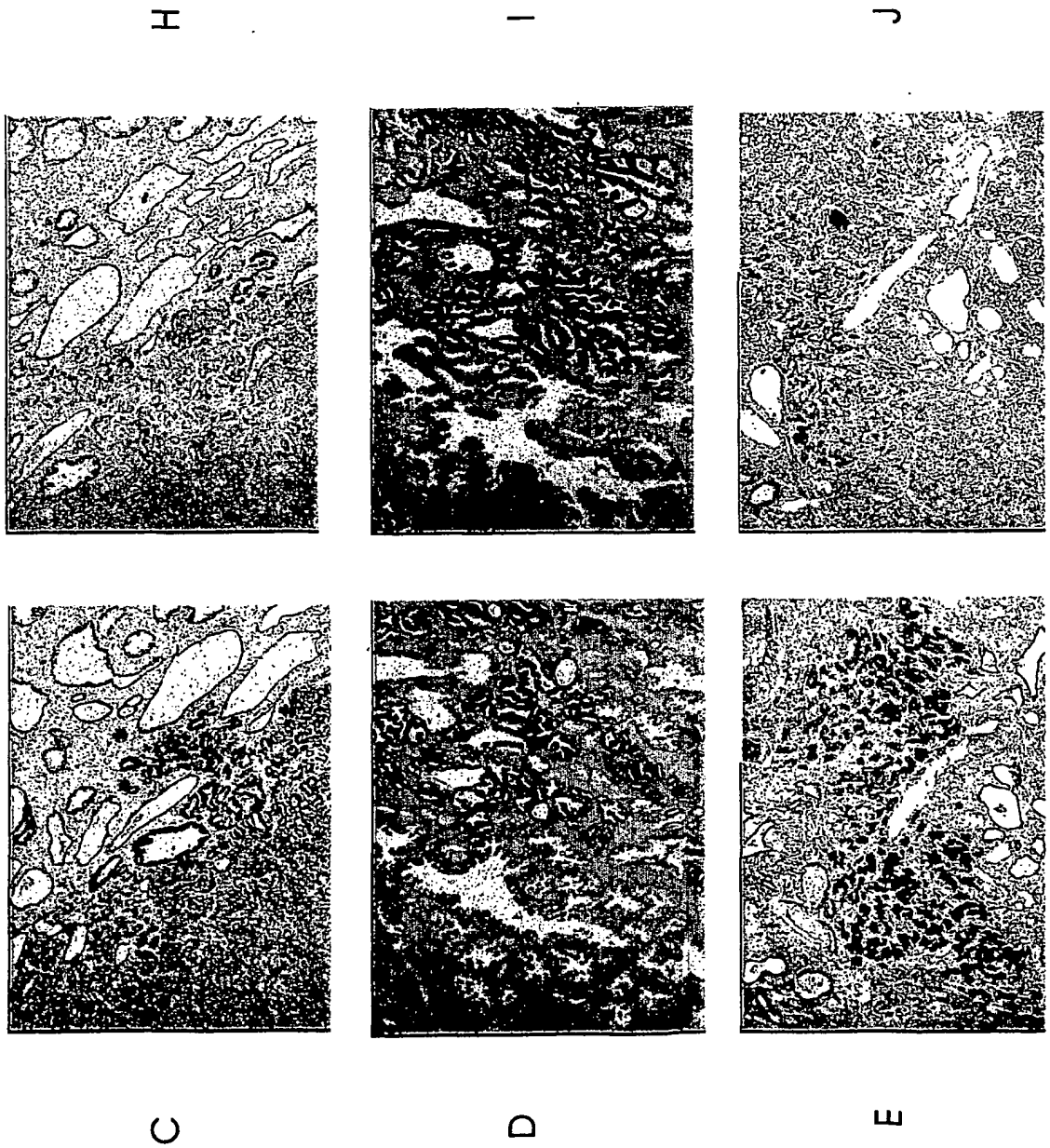


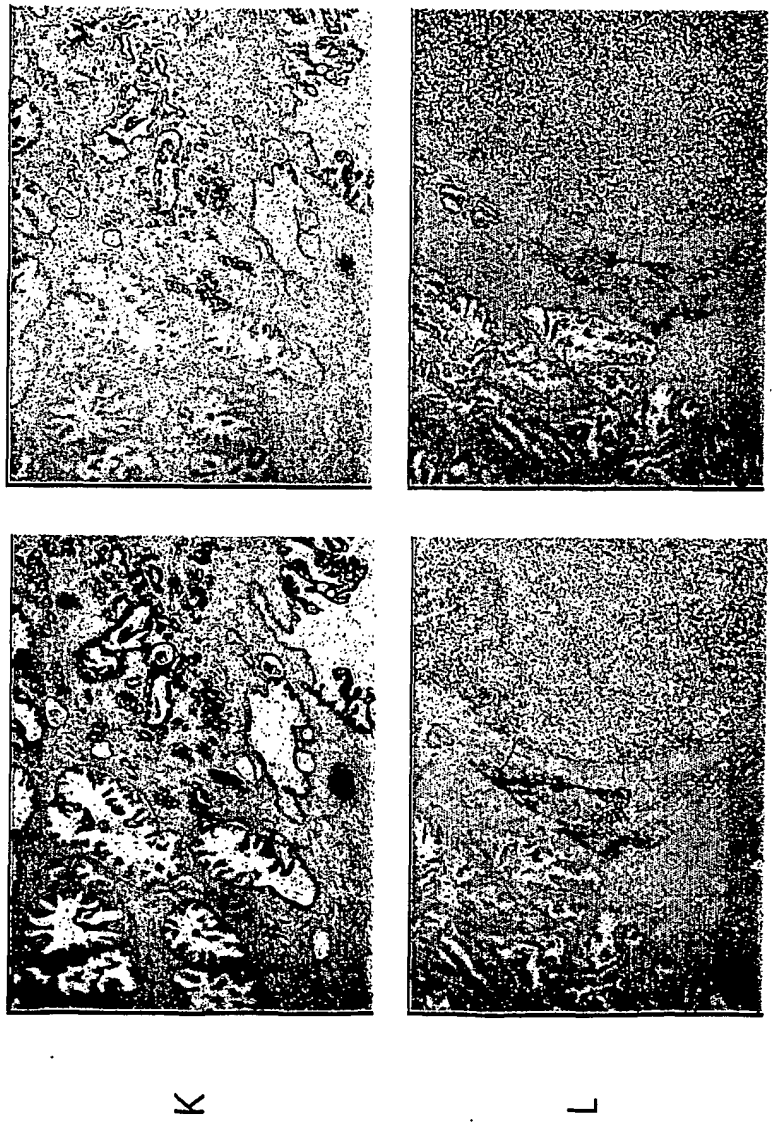
Fig. 13 / Continuation 1



Figs. 14A, 14B, 14F and 14G



Figs. 14C, 14D, 14E, 14H, 14I and 14J



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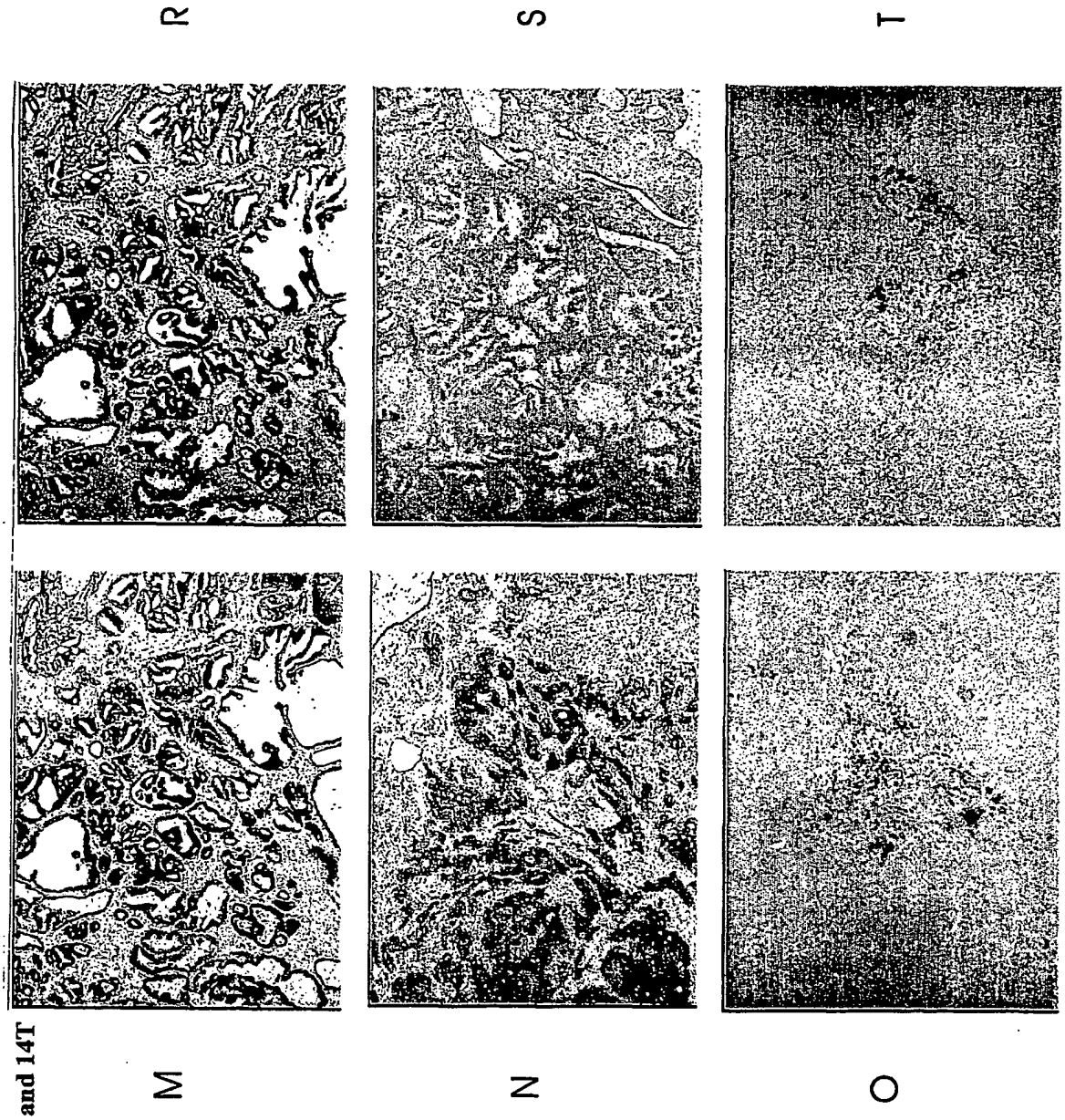
Figs. 14K, 14L, 14P and 14Q



Figs. 14M, 14N, 14O, 14R, 14S and 14T